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Effects of a First Exposure to Ethanol on the Compositions of Neutral and Polar Lipids in *Euglena gracilis* Z, Taken as a Hepatic Cell Model: Equilibration by Citrulline–Malate[1]

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It has been demonstrated in *Euglena* cells that lactate is metabolized along a pathway (1) comparable to that characteristic of hepatic cells (2,3). In addition, the presence of a PEP carboxykinase that is GTP dependent has been established (1). *Euglena* can thus be regarded as a hepatic cell model. It has also been shown that the lipid metabolism of *Euglena* resembles that of animal cells, since it is characterized by high contents of arachidonic, eicopentaenoic, and docosohexaenoic acids (4).

The results of several studies on rat livers have indicated that a chronic consumption of ethanol induces abundant accumulation of fatty acids in the triacylglycerols (5), as well as alterations (6,7) in the phospholipids of the mitochondrial membrane, which affects the enzyme microenvironment and in turn the mitochondrial functions. The alterations in the fatty acid composition of both mitochondrial and microsomal membrane lipids have been studied in detail (8,9) and it has been suggested that ethanol induced the changes in fatty acid desaturase activities. The only study on *Euglena* (10) was very global, since it focused mainly on glucose metabolism.

Citrulline–malate (Stimol) is a complex formed by the stoichiometric combination of DL-malic acid and L-citrulline. The concentration selected for this study is that used on patients for its antiasthenic effects. This dose, applied to a defined culture of *Euglena* (the hepatic cell model), has already been shown to act synergistically on lactate assimilation, without affecting generation time (11).

All studies presented here were carried out on *Euglena* cells grown for generations in lactate medium (control condition) and then submitted, for the first time, to ethanol in a medium in which it constituted the sole carbon source. The

[1] This article is the second of a series using *Euglena* as a hepatic cell model.

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ethanol effects were followed in the absence or presence of the complex (citrulline–malate). Growth kinetics, respiratory intensities, and a detailed study of the accompanying lipid changes have been followed. When compared to the control cells, a beneficial action of citrulline–malate was demonstrated for cells exposed to a strong dose of alcohol.

EXPERIMENTAL

Cell Cultures

The wild-type, Z strain of *Euglena gracilis* was grown organotrophically at 25°C in a mineral medium, pH 3.5 (12), containing 33 mM lactate (control) or 50 mM ethanol as the sole carbon source. The fresh cultures were inoculated at a standardized cell titer of 3×10^4 cells per milliliter. In the ethanol medium, 1.5 mM (0.5 g/liter) citrulline–malate was added (through a sterilized Millipore filter) or not.

Cell Respiration

The respiratory intensity of the cultured cells was polarographically determined using a Clark-type electrode. The measurements were performed each day, directly in the culture medium, in darkness. The results were expressed as micromoles of O_2 consumed per hour and per 10^6 cells.

Lipid Analysis

Lipid extraction. Cell suspensions, containing 3×10^7 cells per milliliter, were centrifuged for 5 min at 1500*g* (Sorvall RC2B), and pellets were resuspended in 8 ml methanol and maintained at 100°C for 20 min. Lipids were extracted with 75 ml of a mixture of chloroform, methanol, and water at a ratio of 8:4:3 (v/v/v). After a strong manual mixing, the emulsion was decanted. The chloroformic phase was evaporated at 35°C and then stored under N_2 at −20°C until separation of the different lipid classes and analysis of fatty acids were carried out on aliquots.

Separation of lipid classes. A spot of chloroformic solution of total lipids was deposited on silica gel TLC plates (kieselgel 60, Merck). A monodimensional separation of polar lipid classes was performed with a mixture of chloroform, acetone, methanol, acetic acid, and water (10:4:2:2:1, v/v/v/v/v). The plate was sprayed with an aqueous solution of rhodamine 6G (0.02%, w/v). The spots corresponding to lipid classes were characterized under ultraviolet light and scraped off the plate.

Esterification and methylation of fatty acids. A volume of 5 ml of methanol, containing 2.5% (w/v) of concentrated sulfuric acid, was added to each of the tubes containing either the extract of total lipids or the silica gel impregnated with a defined lipid class. The tubes were maintained for 1.5 hr at 70°C. The resulting methyl esters were collected in the upper phase after addition of 1 ml of petroleum ether and 1 ml of water (D. Troton, personal communication).

Fatty acid methyl esters (FAME) from each lipid class and from total fatty acids were analyzed by gas chromatography (Delsi 200), using He–U as the

carrier gas in a capillary column (Carbowax 20 M, 0.35 mm × 25 m). The separation was realized at 180°C for 20 min, then at 210°C for 1.5 h; the detector was of flame ionization type (FID).

Quantification and identification. For quantitative analysis, a known quantity of 19:0 (nonadecanoic acid, Sigma) was added as an internal standard. FAME were identified by comparison of their retention times with those of known standards. The identification was also based on the precise determination of the fatty acids specific to *Euglena*, already reported by Troton *et al.* (13). Peak areas were integrated using Enica 21.

Unsaturation indexes. The unsaturation index (U.I.) of a given lipid class was calculated by the average number of double bonds per fatty acid chain (regardless of the number of C atoms), according to the formula (14,15)

$$\text{U.I.} = \frac{\sum_{n=0}^{n=6} (\text{sum of fatty acids composing } n \text{ double bonds}) \times n}{100}.$$

RESULTS

Growth Kinetics

Regardless of the carbon source, it was totally exhausted 84 hr after inoculation. By comparison to the control cells cultured in lactate medium, the replacement of the 33 mM lactate substrate by 50 mM ethanol increased the cell titer by only 16% (Fig. 1). In contrast, the presence of the citrulline–malate complex (1.5 mM) in the ethanol medium increased the final number of cells by 90%. Under all culture conditions, the generation time was consistently 11 hr (Fig. 1). Disappearance of either malate or citrulline from the ethanol (50 mM) medium indicated that the complex acted as a substrate mainly during the transition phase of growth (Fig. 2); its presence did not affect the kinetics of ethanol consumption (Fig. 1).

Respiratory Intensities

For control cells, the respiratory intensities (Fig. 3) were maximal at 48 hr, whereas for cells exposed for the first time to ethanol, the respiratory intensities were in two peaks, at 24 and 60 hr; the presence of the complex enhanced the second peak by 60%.

Variations in the Contents and Distributions of Neutral and Polar Lipids Classes

In the control cells, the contents of total fatty acids decreased as a function of culture time (Fig. 4). At Day 3, the cells that were grown only with the ethanol substrate displayed total lipid contents that increased by a factor of 2 and then regularly decreased. At Day 4, the presence of citrulline–malate, added to the ethanol, depressed the total cell lipids to levels equivalent to those of the control. At Day 8, citrulline–malate depressed the lipids to 60% of the levels of cells grown either on lactate or on ethanol medium.

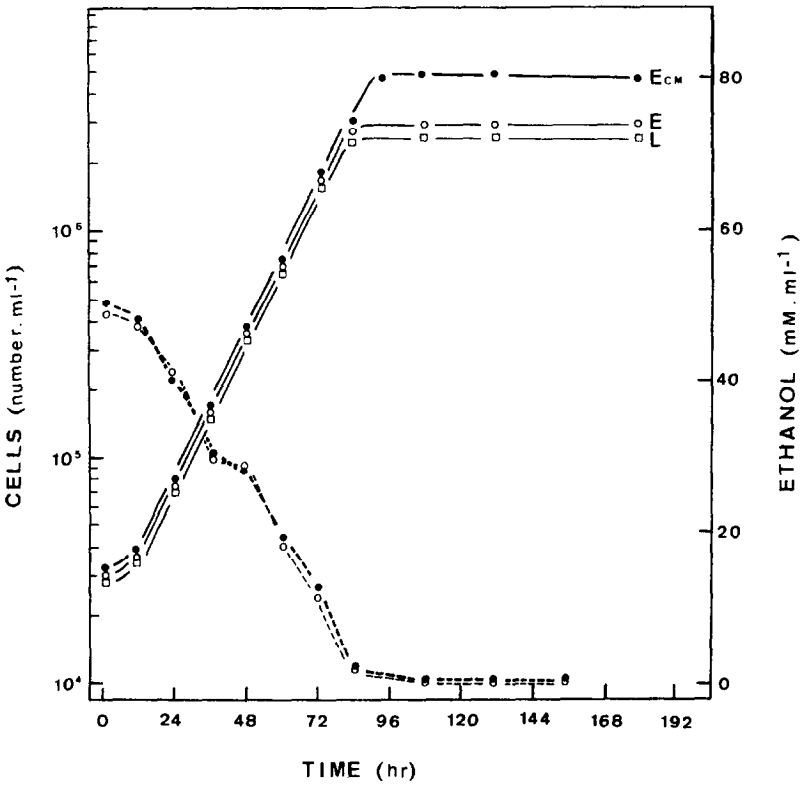

FIG. 1. Growth curves of three cultures of *Euglena* cells inoculated from the same stationary preculture and grown in 33 mM lactate, L (□); 50 mM ethanol, E (○); or 50 mM ethanol plus 1.5 mM citrulline–malate, Ecm (●). In the E and Ecm cultures, the disappearance of the ethanol from the medium is represented by broken lines.

Figure 4 also indicates that the ethanol substrate drastically enhanced the quantities and proportions of cell neutral lipids (NL). The presence of the complex depressed the quantities but not the proportions of cell neutral lipids.

The quantities and distributions of each polar lipid class (PS, phosphatidylserine; PC, phosphatidylcholine; PE + DPG, phosphatidylethanolamine + diphosphoglycerol) are also represented in Fig. 4. The PS level was always very low, representing only 2 to 6% of total lipids. The PC level seemed to be the most affected polar class since (a) its proportions increased from Days 2 to 8, 3 to 8, and 4 to 8 in the respective media: lactate, ethanol, and ethanol plus citrulline–malate; (b) the ethanol, and even more so when the complex was added, diminished the proportions of PC; (c) from Day 3 to Day 8, the presence of the complex drastically decreased the cell content of PC. The results showed that for PE + DPG, whatever the culture day and the substrate used, the distributions remained constant.

Fatty Acid Distributions in the Different Lipid Classes

Table 1 presents detailed data of the fatty acid distributions, higher than 5% and expressed as percentage of the total fatty acids, for each of the five lipid

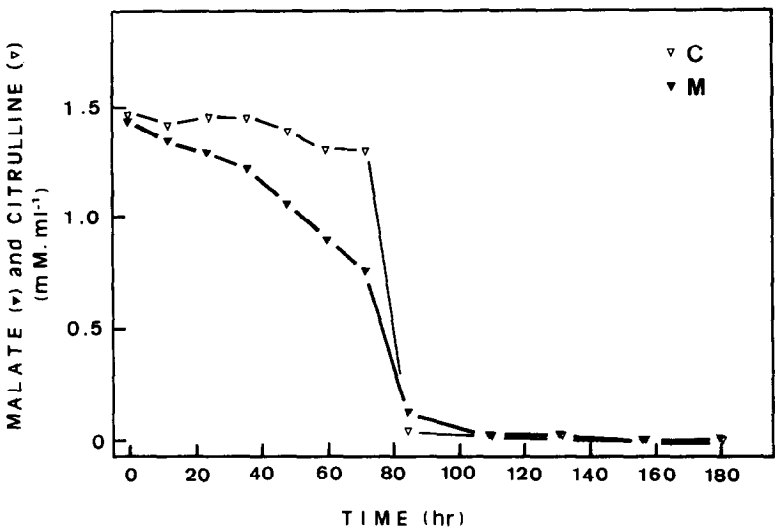

FIG. 2. Disappearance from the ethanol medium, during the culture growth, of each of the components of the 1.5 mM complex: citrulline (▽) and malate (▼).

classes analyzed (PS, PC, PE, DPG, and NL). These distributions are indicated for Days 2, 3, 4, and 8 and for each of the three culture conditions.

The major fatty acids found in the NL were 16:0, 18:0 and 18:1 in the cells cultured in lactate medium and 16:0, 16:4, (*n*-3), 20:4 (*n*-6), and 20:5 (*n*-3) in the cells grown in ethanol medium. The action of the complex was to increase the proportion of 16:4 (*n*-3) and to decrease simultaneously the proportions of 20:4 (*n*-6) and 22:5 (*n*-6). Differences were consequently observed between the U.I. calculated for the three different culture conditions: during the course of growth in lactate medium there was an increase in the U.I., whereas the opposite was true for the cells growing in ethanol medium. The presence of the complex enhanced the U.I. values during the exponential phase of growth and then strongly depressed them during both transition and stationary phases.

PS contained 16:0, 20:4 (*n*-6), and 22:5 (*n*-6) as major fatty acids. The cell distributions changed identically for the three different media as the cultures reached the stationary phase of growth. The 16:0, 20:4 (*n*-6), and 20:3 (*n*-3) decreased when the 22:5 (*n*-6), 18:1, 18:0, and 22:6 (*n*-3) increased. The effect of the ethanol was to reinforce the U.I. compared to that of control cells. The citrulline–malate enhanced the ethanol effect, especially from Days 4 to 8.

PC was characterized by high proportions of unsaturated and long-chain fatty acids (about 75%). During the evolution of a given culture, the distributions of 20:4 (*n*-6), 20:5 (*n*-3), 16:0, and 20:2 (*n*-6) were relatively stable. Ethanol seemed to depress the proportion of 20:5 (*n*-3) to the benefit of 20:3 (*n*-6), 22:5 (*n*-6), and 20:4 (*n*-6), an effect which appeared to be corrected by the addition of the complex.

PE, rich in 16:0, 18:1, and 20:4 (*n*-6), exhibited, as the culture grew, a decrease in the proportions of 16:0 and an increase in the proportions of 18:1. The effect of the ethanol substrate, in the absence as well as in the presence of the complex,

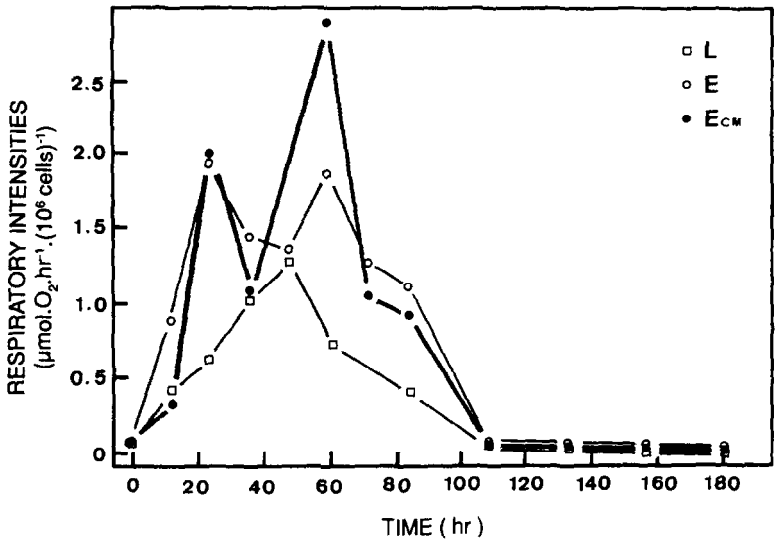

FIG. 3. Respiratory intensities of *Euglena* cells cultured in three different media; lactate (control, □); ethanol (○); ethanol plus citrulline–malate (●). All data are expressed as micromoles of O_2 consumed per hour and per 10^6 cells.

was to intensify the decrease of the 20:4 (*n*-6), to the benefit of the 20:5 (*n*-3) and 22:5 (*n*-6), already observable during the culture growth with lactate. The PE was characterized by S1 = 16:0 + 18:1 + 20:4 (*n*-6), varying from 75% at the beginning to 55% at the end of the culture in lactate; and S2 = 20:5 (*n*-3) + 22:5 (*n*-6) + 22:6 (*n*-3), simultaneously varying from 10 to 28%. On ethanol substrate, either in the absence or in the presence of the complex, the depressed S1 sum varied from 55 to 45%, whereas the reinforced S2 sum increased from 26 to 38%. These evolutions explain the strong increases in the U.I. The citrulline–malate added to the ethanol substrate reinforced the U.I. only transitorily during the exponential phase of culture growth.

DPG, i.e., cardiolipin, present in the lactate grown cells, contained 18:3 (*n*-3), 18:1, 16:0 and 18:0 as major fatty acids. In ethanol-grown cells (a) the 16:0, 18:0, and 18:1 decreased, to the benefit of 20:4 (*n*-6) and 20:5 (*n*-3); (b) from Day 2 to Day 8, the fatty acids 20:5 (*n*-3), 22:5 (*n*-6), and 22:6 (*n*-3) increased, whereas they drastically decreased in the control cells. Compared to lactate substrate, the ethanol depressed the proportions of weakly unsaturated short-chain fatty acids (16:0, 16:1, 16:2, 16:3, and 18:0, 18:1, 18:3) in the cells. In contrast, the ethanol reinforced strongly unsaturated long-chain fatty acids: 16:4, 18:3, 20:4 (*n*-6), 20:5 (*n*-3), 22:5 (*n*-6), and 22:6 (*n*-3). The presence of the complex buffered these ethanol effects, except for the 20:5 (*n*-3) which remained at high concentration. The U.I. of the DPG, which decreased from Day 2 to Day 4 in the cells growing in the lactate medium, constantly increased during growth in ethanol medium. The addition of citrulline–malate enhanced the U.I. values even more.

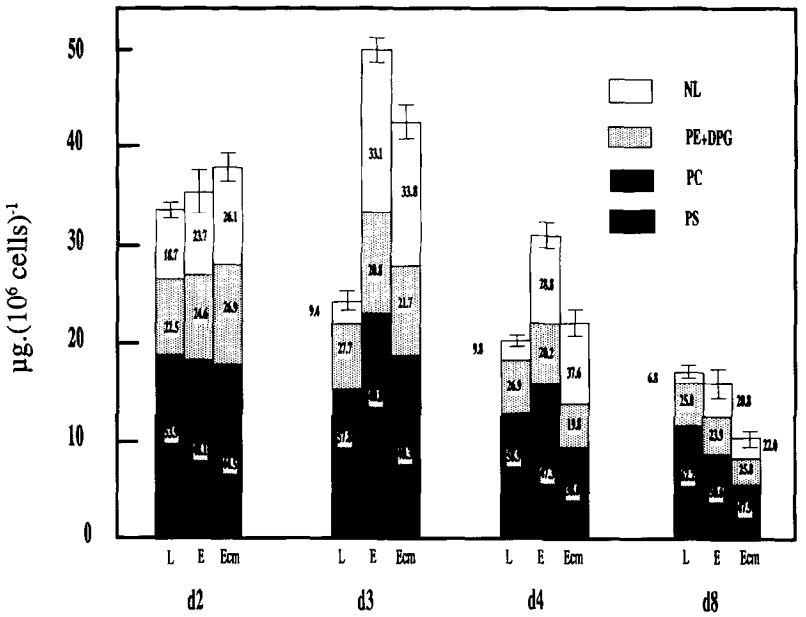

FIG. 4. Fatty acids at Days 2, 3, 4, and 8, expressed as micrograms per 10^6 cells, in the different lipid classes. Data are representative of triplicate experiments. The numbers indicate the distributions, in percentages, of each lipid class: PS (■), phosphatidylserine; PC (■), phosphatidylcholine; PE (□), phosphatidylethanolamine + DPG diphosphoglycerol; NL (□), neutral lipids. The bars, corresponding to the total fatty acids, indicate error levels between ±2.5 and ±9%.

The (n-3) and (n-6) Pathways of Polar Lipids

The (*n*-3) pathway was defined as the sum of 16:3 (*cis*-7,10,13), 18:3 (*cis*-9,12,15), 20:3 (*cis*-11,14,17), 20:4 (*cis*-8,11,14,17), 20:5 (*cis*-5,8,11,14,17), 22:5 (*cis*-7,10,13,16,19), and 22:6 (*cis*-4,7,10,13,16,19), while the (*n*-6) pathway corresponded to the sum of 18:2 (*cis*-9,12), 20:2 (*cis*-11,14), 20:3 (*cis*-8,11,14), 20:4 (*cis*-5,8,11,14), 22:4 (*cis*-7,10,13,16), and 22:5 (*cis*-4,7,10,13,16).

Figure 5 shows, for the individual lipid classes, the fatty acid cellular contents of each of the two pathways. The data are presented for Days 2, 3, 4, and 8 and for each of the three culture conditions. The numbers to the right of each bar indicate, for each of the three lipid classes (PS, PC, PE + DPG), the sum of the percentages of the fatty acids of the polar lipids belonging either to the (*n*-3) pathway (Fig. 5A) or to the (*n*-6) pathway (Fig. 5B). Such a sum of percentages is obviously different from 100.

For the (*n*-3) pathway, compared to that of the lactate substrate, the ethanol depressed the quantities of unsaturated polar fatty acids (Fig. 5A), as well as their distributions. The addition of the complex to the ethanol medium increased the quantities of (*n*-3) polar lipids during the exponential phase of growth, while it decreased these quantities at Days 4 to 8. The effect of the complex was, nevertheless, to reestablish the percentages to those of lactate medium.

For the (*n*-6) pathway (Fig. 5B), the effects of ethanol were opposite those

TABLE 1
Fatty Acid Distributions, in Percentages, of the Total Fatty Acids Present in Each of the Five Lipid Classes (PS, PC, PE, DPG, and NL)

	Fatty acid																	
	16:0	16:1	16:2	16:3	16:4	18:0	18:1	18:2	18:3	20:2	20:3	20:4	20:3	20:4	20:5	22:5	22:6	U.I.
NL																		
L2	27.76	8.92	2.57		0.88	10.93	25.35	4.28		1.36		2.81			1.49	1.27		1.24
L3	27.33	3.96	6.54		10.89	12.95	16.02	6.30		1.66		3.21			3.95	1.15		1.55
L4	31.35	4.30	4.72		6.18	16.27	21.72	7.31		0.93		1.64			2.54			1.06
L8	35.70	4.37	2.95		2.91	17.73	21.48	6.01		0.99		3.31			3.27			0.91
E2	21.82	7.81	2.87		4.15	4.86	11.67	4.30		6.91		11.28			6.79	4.49		2.19
E3	21.64	5.87	8.21		11.08	6.39	8.98	3.10		4.41		11.01			4.25	3.51		2.18
E4	20.34	4.24	14.42		14.39	5.56	6.82	2.26		3.10		9.23			5.12	3.63		2.31
E8	15.53	2.10	5.70		4.82	6.39	6.80	1.46		3.44		20.19			12.41	9.06		2.92
Ecm2	20.37	8.49	2.13		5.31	3.77	9.89	2.07		9.20		10.35			7.55	4.71		2.36
Ecm3	21.22	6.34	7.48		12.98	5.45	9.39	3.09		4.35		8.89			6.31	2.90		2.27
Ecm4	20.39	3.52	17.15		22.43	6.35	5.10	1.33		2.55		5.94			4.64	1.99		2.32
Ecm8	21.37	3.75	5.57		4.28	8.60	17.24	6.17		1.78		11.44			9.31	3.75		2.07
PS																		
L2	30.84					5.24	5.42					20.55	9.64			14.05	2.22	2.27
L3	25.16					5.26	7.92					16.47	8.50			18.07	6.02	2.62
L4	30.14					6.21	6.29					15.03	2.99			22.66	6.03	2.55
L8	25.94					7.53	8.24					9.56	2.58			27.06	5.16	2.54
E2	24.73					3.32	4.31					23.85	8.31			13.36	4.36	2.69
E3	24.12					4.71	3.66					24.06	6.50			17.51	4.70	2.73
E4	23.42					3.03	3.32					20.70	3.43			22.54	6.22	2.98
E8	27.63					7.84	6.21					10.64	2.05			26.84	4.25	2.52
Ecm2	27.93					3.07	4.33					23.11	11.43			11.74	4.43	2.55
Ecm3	23.91					3.43	3.11					24.98	8.68			16.74	6.51	2.89
Ecm4	21.99					3.35	3.04					21.97	4.72			22.94	7.10	3.05
Ecm8	23.53					7.44	7.39					10.01	3.09			29.48	5.98	2.72

PC												
L2	16.14		6.75	7.14	5.87	20.73	5.13	5.10	19.35	4.12	2.38	3.01
L3	12.41		5.45	4.80	6.43	17.00	4.86	8.25	20.15	6.04	5.54	3.38
L4	15.29		4.59	6.70	3.56	18.80	5.35	4.07	19.07	7.33	5.59	3.29
L8	12.91		3.72	7.55	2.54	22.78	7.16	3.33	19.55	7.54	5.40	3.42
E2	11.16		3.71	7.18	10.06	20.68	4.13	7.93	16.95	5.43	3.33	3.33
E3	12.89		3.72	5.17	9.79	23.73	2.44	6.43	14.28	7.24	2.72	3.28
E4	11.92		2.87	4.25	7.48	24.53	3.02	6.06	14.71	10.19	4.34	3.49
E8	10.31		2.74	6.51	3.95	23.54	4.74	5.14	14.78	13.89	6.86	3.63
Ecm2	11.26		4.14	6.47	7.24	16.65	6.63	9.37	22.30	4.04	3.81	3.39
Ecm3	13.47		3.83	4.93	7.73	20.08	3.62	7.86	20.16	5.83	3.40	3.29
Ecm4	12.03		3.64	4.51	6.31	21.17	3.25	6.12	18.43	8.39	5.41	3.48
Ecm8	11.16		2.74	5.68	2.61	22.39	5.03	3.52	18.69	12.20	9.54	3.70
PE												
L2	34.74	6.77	19.89			20.65			2.65	4.52	3.14	1.78
L3	23.99	5.51	22.65			15.50			10.28	8.71	12.28	2.84
L4	22.25	6.29	26.20			14.19			8.50	8.00	8.30	2.48
L8	18.15	2.92	24.97			11.31			9.76	10.02	8.83	2.49
E2	23.34	5.28	17.85			15.24			9.97	6.72	9.42	2.50
E3	19.30	5.39	18.68			17.48			9.78	8.16	8.93	2.63
E4	15.36	5.53	16.08			16.32			13.41	10.78	10.59	3.00
E8	13.96	5.13	23.18			8.77			13.81	13.08	11.40	2.85
Ecm2	24.04	5.21	15.92			15.48			10.95	5.72	12.53	2.64
Ecm3	20.21	5.80	17.75			15.68			11.57	6.05	11.87	2.70
Ecm4	16.76	6.63	17.39			15.54			13.22	7.74	12.12	2.87
Ecm8	12.78	5.81	23.06			7.85			14.93	10.57	13.19	2.88

TABLE 1—*Continued*

	Fatty acid																
	16:0	16:1	16:2	16:3	16:4	18:0	18:1	18:2	18:3	20:2	20:3	20:4	20:3	20:4	20:5	22:5	22:6	U.I.
DPG																		
L2	19.34	6.77	0.77	0.61	0.58	14.79	12.14	3.16				0.88			20.15	4.51	14.66	2.49
L3	14.76	6.55	7.26	14.16		7.82	14.11	9.53	19.73			1.68			2.03			1.80
L4	21.43	7.05	2.63	14.38	2.86	8.13	15.17	4.37	17.44			3.26			1.90	0.92	0.46	1.73
L8	18.66	6.16	7.03	8.95		9.77	15.55	10.14	13.43			3.49			3.77	0.99	0.49	1.69
E2	15.02	5.95	2.18	11.23	6.46	10.43	9.92	4.65	24.63			2.70			2.45	0.90	0.73	2.03
E3	9.06	4.88	2.75	16.89	4.23	2.68	8.38	3.56	26.96			7.77			4.40	1.96	1.20	2.61
E4	10.88	4.31	1.71	5.05	1.47	2.04	9.60	2.64	8.64			19.68			11.60	6.75	3.49	2.99
E8	11.51	4.88	3.11	3.86	0.86	3.01	10.03	2.83	6.17			18.06			12.47	8.02	4.52	2.94
Ecm2	14.91	4.87	2.62	10.41	9.27	4.57	11.14	4.28	27.74			2.85			2.74	1.04	0.94	2.25
Ecm3	10.47	4.21	1.63	10.03	5.32	2.22	8.74	2.36	18.88			11.18			10.05	2.62	2.48	2.83
Ecm4	9.23	4.72	1.73	6.03	2.26	1.57	8.71	2.59	9.93			16.47			14.77	4.81	4.71	3.11
Ecm8	11.66	4.23	1.68	3.68	0.97	2.88	9.51	2.26	5.06			17.26			15.77	7.15	6.78	3.09

Note. These distributions are indicated for Days 2, 3, 4, and 8 for each of the three types of culture: L, lactate; E, ethanol; Ecm, ethanol + citrulline–malate. Unsaturation indexes (U.I.) are also indicated.

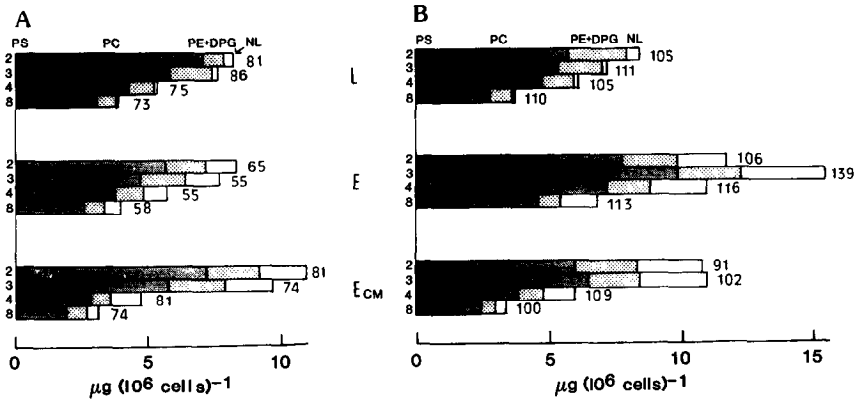

FIG. 5. Fatty acid contents belonging to (*n*-3), A, and (*n*-6), B, pathways in the different lipid classes: PS (■), PC (▣), PE + DPG (□), and NL (□). Cells were grown in the respective media: L, lactate; E, ethanol; Ecm, ethanol + citrulline-malate. The lipid contents were analyzed at Days 2, 3, 4, and 8 for each of the cultures. The numbers at the right side of the bars indicate the sum of the polar lipid proportions, only relative to the (*n*-3) and (*n*-6) pathways. All data are representative of triplicate experiments.

analyzed for the (*n*-3) pathway. There was a reinforcement of the quantities by a factor 1.5. The addition of the complex to the ethanol medium affected the contents more than the proportions of the (*n*-6) polar fatty acids.

DISCUSSION

In the experiments presented in this paper, *Euglena* cells previously grown in lactate medium were exposed for the first time to ethanol; for the first 84 hr, six cell generations occurred and then the culture entered the stationary phase. Analyses were performed on Days 2, 3, 4, and 8. The number of carbon atoms was identical in the 33 mM lactate medium, considered the control, and in the experimental 50 mM ethanol medium. This explains the similar kinetics of culture growth (Fig. 1). However, the ethanol substrate enhanced the final cell titer by 16 and by 90% when the citrulline–malate was added, which accounted for only 15% of additional exogenous carbon. This suggests important modifications in the metabolic pathways.

The results (Fig. 2) indicate that the citrulline and the malate did not act as competitive substrates relative to ethanol; in fact, their consumptions were belated since they took place mainly during the transition phase of culture growth, a time at which the ethanol was almost exhausted. The complex induced a strong increase in the respiratory intensities (Fig. 3). A preliminary study (data not shown) with *Euglena* cells cultured in 50 mM ethanol supplemented with the individual components of the complex (citrulline or malate, 1.5 mM) indicated that the citrulline was responsible for the last cell doubling, whereas the malate played the role of the respired energetic substrate.

Since the cell reserves that accumulate from the ethanol are mainly lipids—when it is β-1, 3-glucan, paramylon, in cells grown with lactate substrate (1,11)—

TABLE 2
Modifications, at Day 3, of Parameters Relative to Fatty Acid Compositions of Membrane Phospholipids

Ratio	Phospholipid	Culture media		
		Lactate	Ethanol	Ethanol + CM
18:1/16:0	PS	0.31	0.15+	0.13
	PC	0.44	0.29+	0.28
	PE	0.94	0.96	0.88
	DPG	0.95	0.92	0.83
20:4/16:0	PS	0.65	0.99	1.04
	PC	1.36	1.84+	1.49*
	PE	0.64	0.90+	0.77*
	DPG	0.11	0.85	1.06
20:4(*n*-6)/18:2(*n*-6)	PS	11.51	20.21+	25.49
	PC	17.00	31.22+	36.50
	PE	6.27	5.75	7.06
	DPG	0.17	2.18+	4.73
20:4(*n*-6)/20:5(*n*-3)	PS	5.14	12.53+	7.57*
	PC	0.84	1.66+	0.99*
	PE	1.50	1.78+	1.35*
	DPG	0.82	1.77+	1.11*
22:5(*n*-6)/22:6(*n*-3)	PS	3.00	3.72+	2.57*
	PC	1.09	2.66+	1.71*
	PE	0.70	0.91	0.50
	DPG	2.00	1.63	1.05

Note. +, significant effect of ethanol; *, significant effect of citrulline–malate. The values of the ratios were calculated from percentages in the considered class.

the focus of this study was the analysis, according to the type of culture medium, of the changes in total fatty acid contents and in fatty acid compositions of the different lipid classes. The main effects of the first exposure of the cells to ethanol were (a) important changes in total fatty acid contents (Fig. 4), (b) increases in neutral lipid levels (Fig. 4), and (c) variations in the fatty acid compositions (Table 1) of the polar lipids (PS, PC, PE, and DPG).

The results of an ethanol feeding of *Euglena* were a decrease in 16:0 in all lipid classes (Table 1) and an increase in the production of unsaturated long-chain fatty acids (20:4, 20:5, 22:5, and 22:6), which consequently enhanced the U.I. values. Thus, the resulting calculated 18:1/16:0 ratio (Table 2) was found to decrease in both PS and PC phospholipids, indicating reinforcement of the elongation of palmitic acid followed by the desaturation of the resulting stearic acid. For all the polar lipids, except the PE class, a strong increase in arachidonic acid was observed, indicating that the desaturation system, which includes the Δ6 and Δ5 desaturases, may be activated by ethanol. The 20:4/16:0 ratio was hence found enhanced (Table 2) in all the studied phospholipids, and the 20:4/18:2 ratios were multiplied by a factor of 2 in both PS and PC (Table 2).

In all the classes, the 20:4/20:5 and 22:5/22:6 ratios increased dramatically, by reinforcement of the numerators, when the cells were fed with ethanol, in comparison to the control cells fed with lactate; these data suggest that the ethanol deactivates the Δ17 and Δ19 desaturases (Table 2). In the *Euglena* cells, the (*n*-3) and (*n*-6) pathways were therefore the most affected by the ethanol (Fig. 5).

These ethanol effects on *Euglena* cells were globally in agreement with those observed by Nervi *et al.* (5) on the liver triacylglycerols and by Cunningham *et al.* (8) on the fatty acid compositions of the microsomal phospholipids. The latter authors proposed an increase in the activity of Δ9 desaturase to explain the decrease in 16:0 to the benefit of the 18:1. In contrast, Umeki *et al.* (16), investigating the fatty acid desaturase activities of liver microsomes extracted from ethanol-fed rats, have shown that the Δ9 desaturase activities drastically decreased and that the NADH + H^+/NAD^+ ratio increased over two-fold. These authors then hypothesized that the high NADH + H^+ level would stimulate the biosynthesis of triacylglycerols. According to (17), the increase of NADH + H^+ content would result from, on the one hand, enhanced alcohol dehydrogenase activity and, on the other hand, decreases in the oxidation rate of NADH + H^+ and in the activities of NAD(H)-dependent enzymes, such as fatty acid-CoA desaturases. The 20:4/18:2 ratio characterizing the hepatic desaturation–elongation system was the lowest in ethanol-fed rats (18) and indicated a decrease in the Δ6 and Δ5 desaturase activities (9).

Differences as well as similarities then exist between *Euglena* and rat liver. As differences, one can note that in *Euglena*, the 18:0 and 18:2 fatty acids were never a majority, while the 20:5, 22:5, and sometimes the 22:6 were present at high concentrations. Hence, the desaturase activities of *Euglena* cells were opposite those reported for rat liver (16,9). Another difference is that *Euglena* cells are able to synthesize their own linoleic acid, 18:2, the precursor of arachidonic acid, and that when cultured in a defined medium, the composition of arachidonic acid is consequently remarkably constant, thereby allowing a very reliable metabolic study. In contrast, for rats unable to synthesize their own 18:2, the fat diets, which varied from one experiment to another, generated levels of arachidonic acid and desaturase activities that were highly variable and also dependent on the sex of the rat (8).

As similarities, we believe, in agreement with Cunningham *et al.* (8), that the distributions of the phospholipid classes were almost unaffected by ethanol feeding, whereas the compositions in percentages of several fatty acids in a particular class were strongly changed. The PC and DPG classes were the most affected. The linoleic acid levels in DPG (cardiolipin) decreased (Table 1): this observation is in agreement with the analyses of Waring *et al.* (19) on the fatty acid compositions of mitochondrial cardiolipin from rat liver. The results presented in this paper, which do not distinguish between mitochondria and microsomes, still indicate strong similarities between mitochondria from *Euglena* cells and those from rat liver, since the cardiolipin was demonstrated to be synthesized and stored only in the mitochondria (20).

The effects of the complex take place primarily at the level of the elongation–desaturation system within each of the lipid classes. The citrulline–malate added

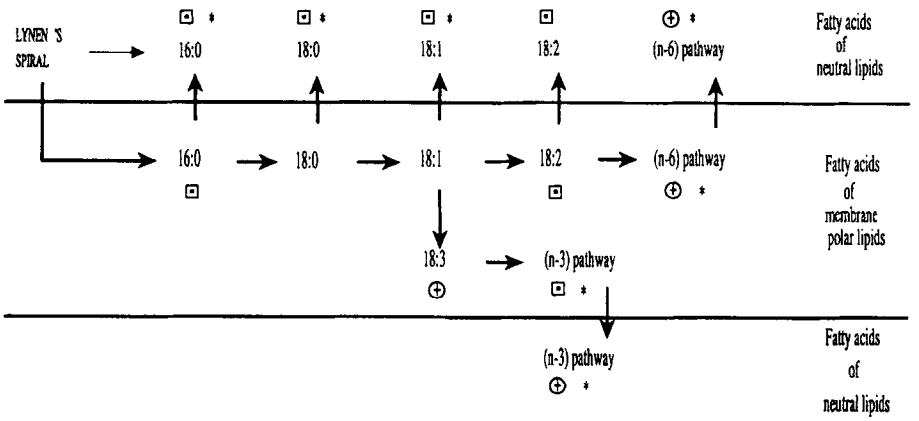

FIG. 6. Effects of ethanol on the lipid metabolism. ⊕ and ⊖ were positive and negative effects of ethanol. *Equilibration to lactate situation by the citrulline-malate complex.

to the ethanol equilibrates the fatty acid concentrations of the (*n*-6) pathway (Fig. 5), especially in the most affected PC class, to those characterizing the *Euglena* cells growing in lactate medium. Another result is that both the 20:4/20:5 and the 22:5/22:6 ratios, enhanced in the presence of only ethanol, became close to those of lactate-grown cells (Table 2). Consequently, the complex seems to regulate the elongation–desaturation system. The citrulline–malate decreases the cellular ATP content (results not published), which explains the diminished quantities of unsaturated fatty acids observed by Israel *et al.* (21) and Thompson and Reitz (9), which correlate the chain elongation with the cellular levels of ATP initially activating the fatty acids.

The complex also induces a delipidation of the cells. On the one hand, at Day 8, during the last cell division, the lipid contents were reduced by a factor of more than 2. On the other hand, the content of neutral lipids was reduced, an effect that could be attributed to the malate and that would favor the reoxidation of the cytosolic NADH + H^+ and hence diminish the biosynthesis of triacylglycerols (16).

The results presented in this paper differ from those of Beaugé *et al.* (22), who had demonstrated that the addition of amino acids to the culture medium of hepatocytes, isolated from rat, accelerated the ethanol catabolism. However, the presence of citrulline–malate, totally exhausted from the medium (Fig. 2), did not induce any modification in the kinetics of ethanol disappearance (Fig. 1). Our data show that it is only when 75% of the ethanol (Fig. 1) is consumed that the citrulline is metabolized (Fig. 2). Either the ethanol substrate modifies the plasma membrane permeability and favors the entry of citrulline or, because the ammonium from the medium is exhausted (data not shown), the citrulline acts as ammonium donor and modifies the subsequent cell metabolism. In support of the first hypothesis, when the ethanol is actively metabolized, the PC is strongly enhanced and the PE is more or less unaffected. Later, at Day 4, as a consequence of the complex penetration, the proportions of polar lipids are

equilibrated to those of the lactate situation (Fig. 5). Since it is known that the unsaturated PC stabilizes the bilayer configuration, while the unsaturated PE does not (23), important changes in PC affect the membrane integrity. In support of the second hypothesis, experiments involving ethanol supply between Days 3 and 4 in the absence or presence of the complex will be undertaken.

We summarize in one recapitulative scheme (Fig. 6) the results of this lipid study on *Euglena*. We present the modifications induced by the ethanol used as substrate in place of the lactate and emphasize the equilibrating effect of addition of citrulline–malate, a complex used for its antiasthenic effects. Figure 6 indicates that the ethanol depresses the 16:0 to the benefit of the (*n*-6) pathway on the one hand and of the 18:3 precursor of the (*n*-3) pathway on the other hand. Following the action of phospholipases, induced by ethanol, which eliminate the polar radical and liberate the neutral lipids, the (*n*-3) fatty acids do not remain in the membranes. The effect of the complex is to equilibrate the fatty acids to levels which characterize the lactate situation.

SUMMARY

In comparison to the lipid composition of *Euglena* cells fed with lactate, a first exposure of the cells to ethanol favors the production of neutral lipids containing mainly unsaturated fatty acids. The ethanol diminishes drastically the proportion of PC and weakly that of PE. In contrast, it increases slightly the proportion of DPG. The ethanol induces important changes in the fatty acid distributions of each lipid class, suggesting modifications of the elongation–desaturation system. On the one hand the proportion of unsaturated fatty acids is increased and, on the other hand, the last double bond is predominately situated in the Δ6 position in place of Δ3. The addition of the complex citrulline–malate corrects most of these changes.

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