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Disturbed sugar metabolism in a fully habituated nonorganogenic callus of Beta vulgaris. Additional data

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RÉSUMÉ

CARRIE, B., B. BISBIS, C. PENEL, Th. GASPAR & H. GREPPIN (1995). Perturbation du métabolisme glucidique dans des cals nonorganogènes totalement habitués de Beta vulgaris. Données supplémentaires. *Saussurea* 25: 143-151. En anglais, résumés français et anglais.

Chez la betterave à sucre, les cellules de cals blancs non organogènes totalement habitués (HNO) absorbent plus d'oxygène que les cellules de cals normaux (N) verts mais dégagent moins de CO_2. Les échanges gazeux par les cellules N sont rythmiques et amplifiés par l'addition de glucose, ce qui n'est pas mis en évidence chez les cellules HNO. Les cellules HNO présentent un nombre plus élevé d'isoformes de malate et alcool déshydrogénases. Ces dernières pourraient confirmer une voie de fermentation privilégiée chez les cellules HNO. Dans les cals HNO, l'augmentation de matière sèche liée au sucrose est toujours plus élevée (± 80%) que celle qui dépend de la fixation du CO_2 (± 20%). L'auxotrophie de 20% des cals HNO rend compte de la fixation de CO_2 non-photosynthétique, probablement par le phosphoenolpyruvate, ce qui expliquerait les activités plus élevées de la malate déshydrogénase, les activités redox liées à NAD/NADH, et l'accumulation de composés azotés. L'apparition précoce de nécroses dans les cals HNO indiquerait la présence de quinones provenant de la chaîne oxydative extra-mitochondriale. Avec l'augmentation, montrée précédemment, de la voie des pentoses phosphates, les cellules HNO déficientes en cytochrome et mitochondries seraient ainsi caractérisées par plusieurs déviations du métabolisme glucidique, comme le montre la figure 3.

ABSTRACT

CARRIE, B., B. BISBIS, C. PENEL, Th. GASPAR & H. GREPPIN (1995). Disturbed sugar metabolism in a fully habituated nonorganogenic callus of Beta vulgaris. Additional data. *Saussurea* 25: 143-151. In English, French and English abstracts.

White fully habituated nonorganogenic (HNO) sugar beet callus cells absorbed more oxygen than green normal (N) callus cells but evolved less CO_2. Gas exchanges by N cells were rythmic and amplified by glucose addition, which was not evident for HNO cells. HNO cells exhibited a higher number of malate and alcohol dehydrogenases isoforms. The latter might confirm a privileged fermentation pathway in HNO cells. The sucrose depending dry matter increase of the HNO callus was always higher (± 80%) than this depending upon CO_2 fixation (± 20%). The 20% auxotrophy of the HNO callus accounted for non-photosynthetic CO_2 fixation, probably through phosphoenolpyruvate, which might account for the higher malate dehydrogenase activities, the

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higher NAD/NADH dependent redox activities, and the accumulation of nitrogenous compounds. Early appearance of necrosis bands in HNO callus might be indicative of quinones generated through an extra-mitochondrial oxidative chain. With the previously shown enhancement of the pentose phosphate pathway, the cytochrome and mitochondria deficient HNO cells thus might be characterized by several deviations of sugar metabolism as tentatively schematized in Figure 3.

Introduction

Cells from a fully habituated (auxin- and cytokinin-independent) nonorganogenic sugar beet callus (HNO) line have been recognized as true cancer cells (GASPAR & al., 1991). They indeed show morphological and biochemical traits similar to animal cancer cells (HAGÈGE & al., 1992a; CRÈVECOEUR & al., 1992). They namely exhibit abnormal mitochondrial structures (CRÈVECOEUR & al., 1992) and an alteration in the control of the tetrapyrrole metabolism with a deficiency in cytochromes (HAGÈGE & al., 1992b). Deviations of sugar metabolism have been shown in animal cancer cells (ALBERTS & al., 1989). In contrast to cells from a normal (N) (auxin- and cytokinin-dependent) callus line, HNO cells accumulate glucose and fructose, and show an abnormal high fructose/glucose ratio. Moreover, HNO cells display lower glycolytic enzyme activities (hexose phosphate isomerase and phosphofructokinase) which is compensated by higher activities of the enzymes of the hexose monophosphate pathway (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) (BISBIS & al., 1993). We report here results of different types of experiments which allow to further characterize the sugar metabolism of these HNO cells. Their gas (O_2, CO_2) exchanges have been compared to those of N cells, in the absence and in the presence of glucose. Their malate and alcohol dehydrogenase activities have also been measured. Their capacities to incorporate ^{14}C-sucrose and their ways to utilize it finally were determined.

Material and methods

Plant material and cultures

Experimental conditions for obtaining N and HNO calli of sugar beet (*Beta vulgaris* L. *altissima*) and for maintaining these tissues in solid stock cultures under light (16 hrs. photoperiod of Sylvania Grolux fluorescent light providing 17 W m^{-2}, 25°C) have been reported elsewhere (DE GREEF & JACOBS, 1979; KEVERS & al., 1981). Calli were subcultured every three weeks on their respective solid medium (basal medium without growth regulators in the case of the HNO line, but supplemented with 0.1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} BAP for the N line), in the presence of 1 or 3% sucrose. Before weighing, the cells were placed on an absorbent filter paper in order to drain the excess of extracellular water. This operation removed 25% of the initial weight for N cells and 33% for H cells.

Respiration

Gas (O_2, CO_2) exchanges were measured using the classical Warburg respirometer and related analytical methods.

^{14}C-sucrose incorporation

The experimental set up developed by DE RIEK & al. (1991) was adapted by BISBIS & al. (1994a). ^{14}C-sucrose was added to the culture media. Decrease of the radioactivity of the media, incorporation in the calli and the release of $^{14}CO_2$ were followed by sampling three jars weekly over a culture period of three weeks. For $^{14}CO_2$-captation the head space of each jar was provided with a plastic cuvette containing 1 ml of NaOH 1 M in which a strip of filter paper was immersed. Measurements of radioactivity were done according to BISBIS & al. (1994a).

Enzymatic determinations

Cells (1 g of drained material) were ground in a small mortar kept on ice in 4 cm^3 of 100 mol m^{-3} Tris-HCl buffer, pH 7.8, containing 10 mol m^{-3} $MgCl^2$, 5 mol m^{-3} dithiotreitol, and 500 mg of insoluble polyvinylpyrrolidone (Polyclar AT). The resulting extract was centrifuged at 27,000 *g* for 20 min. The proteins present in extracts prepared as described above were separated by isoelectric focusing using Servalyt precotes, pH 3-10 (Serva), according to the instructions of the manufacturer. Before separation, the extracts were concentrated from 2 to 0.1 cm^3 with Centricon-10 (Amicon) microconcentrators. The concentrated extracts were diluted to 2 cm^3 with distilled water, concentrated again to 0.1 cm^3 and diluted to 1 cm^3 with water. Samples corresponding to 25 mg proteins (determined by the method of BRADFORD (1976) using bovine serum albumin as a standard) were layered directly on the gel. At the end of the electrophoresis, the gels were stained for various dehydrogenase activities according to the methods described by FINE & COSTELLO (1963) adapted by CARRIÉ & al. (1994).

Results and discussion

Gas exchanges

Oxygen consumptions and carbon dioxide emission by N and HNO cells in suspensions have been measured at days 7, 8 and 13 (Table 1, Fig. 1). Mean oxygen consumptions by N and HNO cells were of about the same value orders but somehow higher for HNO cells. Oxygen uptake by N cells was generally increased by the addition of glucose in the bathing media but that of HNO cells practically unchanged (Table 1). The respiration rythmicity was particularly marked in N cells as compared to HNO cells (Fig. 1). CO_2 emission by N cells was somehow higher than that of HNO cells (Fig. 1). N cells generally responded to glucose addition by an enhanced CO_2 emission; HNO cells did practically not. Here again, the rythmicity of CO_2 emission was well evidenced and even amplified in N cells. This difference in CO_2 evolvement by N and HNO cells induced a different CO_2/O_2 ratio (Table 1) with a rythmicity for N cells (Fig. 1). The absence of response of HNO cells to glucose addition may be related to glucose accumulation in these cells under current condition (BISBIS & al., 1993), or to the low activity and potential of the tricarboxylic acid (Krebs) cycle. The higher CO_2 emission, and the response of N cells to glucose in contrast expresses a higher activity and capacity of the Krebs cycle. The rythmicity of O_2 and CO_2 exchanges by N cells, and the correspondence of high O_2 uptake with high CO_2 emission, reflects the good equilibrium and cooperation existing in these cells between the aerobic fermentation pathway and the aerobic glycolysis-Krebs one,

which is less evident for the HNO cells. The gas exchange ratios, as seen from Figure 1 and Table 1, well illustrate the different modulations of Pasteur and Crabtree effects in N and HNO calli, with a paradoxal anaerobic metabolism type of HNO cells under aerobic conditions.

Enzyme activities

Calli extracts where higher activities of glucose-6-phosphate dehydrogenase and malate dehydrogenase were shown formerly for HNO cells (BISBIS & al., 1993; CARRIÉ & al., 1994), were submitted to gel isoelectric focusing. The gels were stained for various dehydrogenases, including malate dehydrogenase, alcohol dehydrogenase, glutamate dehydrogenase, glucose-6-P-dehydrogenase, and isocitrate dehydrogenase. In the conditions used, only malate and alcohol dehydrogenases gave a good reaction (Fig. 2), the other ones yielding only a very faint staining. Isoelectric focusing separation of malate dehydrogenase isoforms showed that the two cell lines contained almost the same isoenzymes and confirmed the presence of a much higher activity in HNO cells. Alcohol dehydrogenase also appeared with numerous isoforms. There was one main isoenzyme which was somewhat more active in N cells, but HNO cells exhibited more minor bands which may indicate a higher alcohol dehydrogenase activity. Malate plays different prominent roles in cell physiology (LANCE & RUSTIN, 1984). It works as a redox shuttle and is mainly involved in carbohydrate and lipid breakdown. Malate is involved in resistance to anoxia under flooding conditions through "malate fermentation" (LANCE & RUSTIN, 1984). HNO cells, as a hyperhydric (vitrified) tissue (GASPAR & al., 1991; LE DILY & al., 1993), might be considered as being in such conditions. Malate dehydrogenase is not restricted to mitochondria and to its role in the Krebs cycle but is present at different cellular sites. Its role in sugar catabolism by HNO cells needs further investigation. Higher activity of alcohol dehydrogenase however should be indicative of fermentation.

Sucrose utilization

A former study from BISBIS & al. (1994a) on sucrose uptake and metabolism has allowed to calculate the dependency of dry matter increase of both callus types upon sucrose versus CO_2 fixation on the one hand, and to evaluate the repartition of the uptaken sucrose between growth increment and respiration (Table 2). The sucrose depending dry matter increase of the HNO callus was always higher (± 80%) than this depending upon CO_2 fixation (± 20%). The dry matter increase of the green N callus depended much more upon CO_2 fixation (± 40%). Sucrose absorption served approximately 50% for growth and 50% for respiration in the HNO callus whatever the conditions were. The N callus utilized the adsorbed sucrose more for its respiration than for its growth.

		N		*HNO*	
	Days	*glucose —*	*glucose +*	*glucose —*	*glucose +*
O_2 µmol/g.FW/10 min	8	12.28 ± 7.39	17.77 ± 5.53	20.39 ± 8.82	20.74 ± 7.77
	13	13.23 ± 7.11	14.67 ± 7.95	19.69 ± 7.16	20.05 ± 7.87
CO_2/O_2	8	3.58 ± 0.35	1.62 ± 0.40	1.39 ± 0.31	1.49 ± 0.45
	13	4.06 ± 0.88	2.26 ± 0.69	1.49 ± 0.36	1.46 ± 0.55

Table 1 — O_2 uptake and CO_2/O_2 ratio of 8- and 13-day old N and HNO cells in the absence and in the presence of 1 mM glucose.

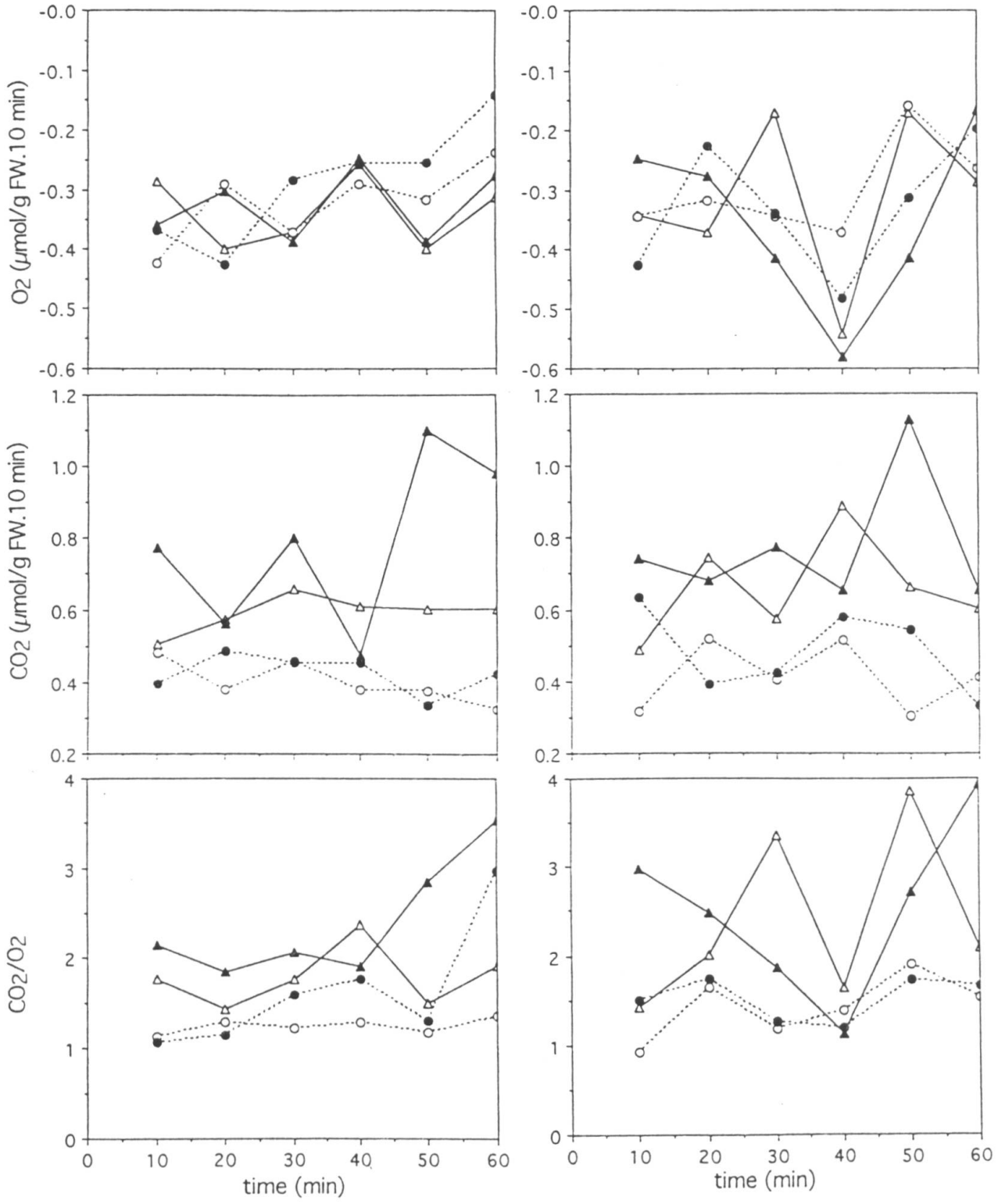

Fig. 1. — O_2 uptake, CO_2 emission and CO_2/O_2 ratio of normal (▲, △) and habituated cells (●, ○) in the absence (△, ○) and in the presence (▲, ●) of 1 mM glucose, at day 7 (left) and 13 (right).

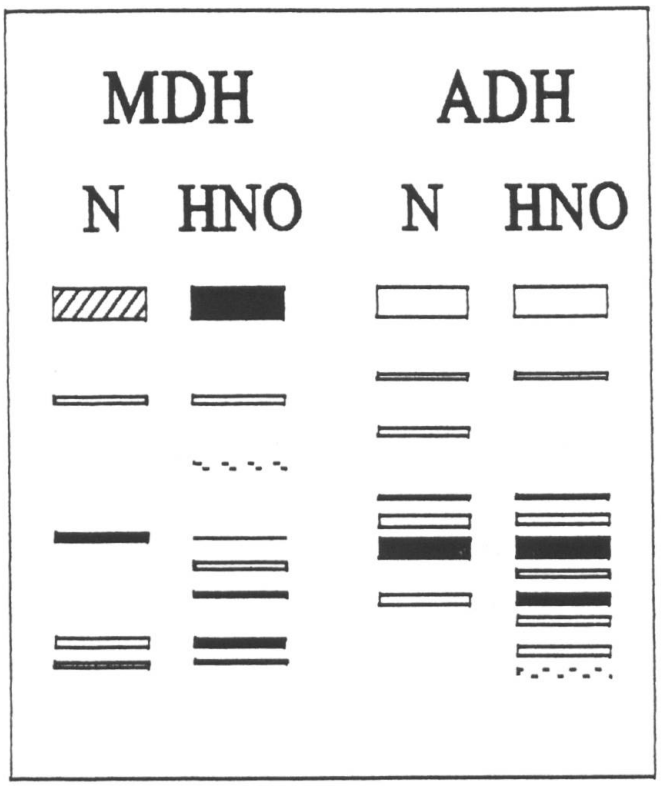

Fig. 2. — Malate (MDH) and alcohol (ADH) dehydrogenase isoforms of 13 day-old N and HNO calli after electrofocusing.

	N		*HNO*	
	sucrose 1%	*sucrose 3%*	*sucrose 1%*	*sucrose 3%*
DM increase mg C. wk^{-1}. jar^{-1}				
— From sucrose (%)	62.28	56.34	76.06	80.37
— From CO_2 fixation (%)	37.82	43.66	23.94	19.63
Sucrose uptake mg C. wk^{-1}. jar^{-1}	40.08	162.06	41.28	107.65
— For growth (%)	44.36	36.97	51.50	49.99
— For respiration (%)	55.63	63.03	48.50	50.01

Table 2. — Carbon balance of sugar beet N and HNO calli, in the presence of 1 or 3% sucrose, calculated from the results of uptake and metabolism of ^{14}C-sucrose (BISBIS & al., 1994a).

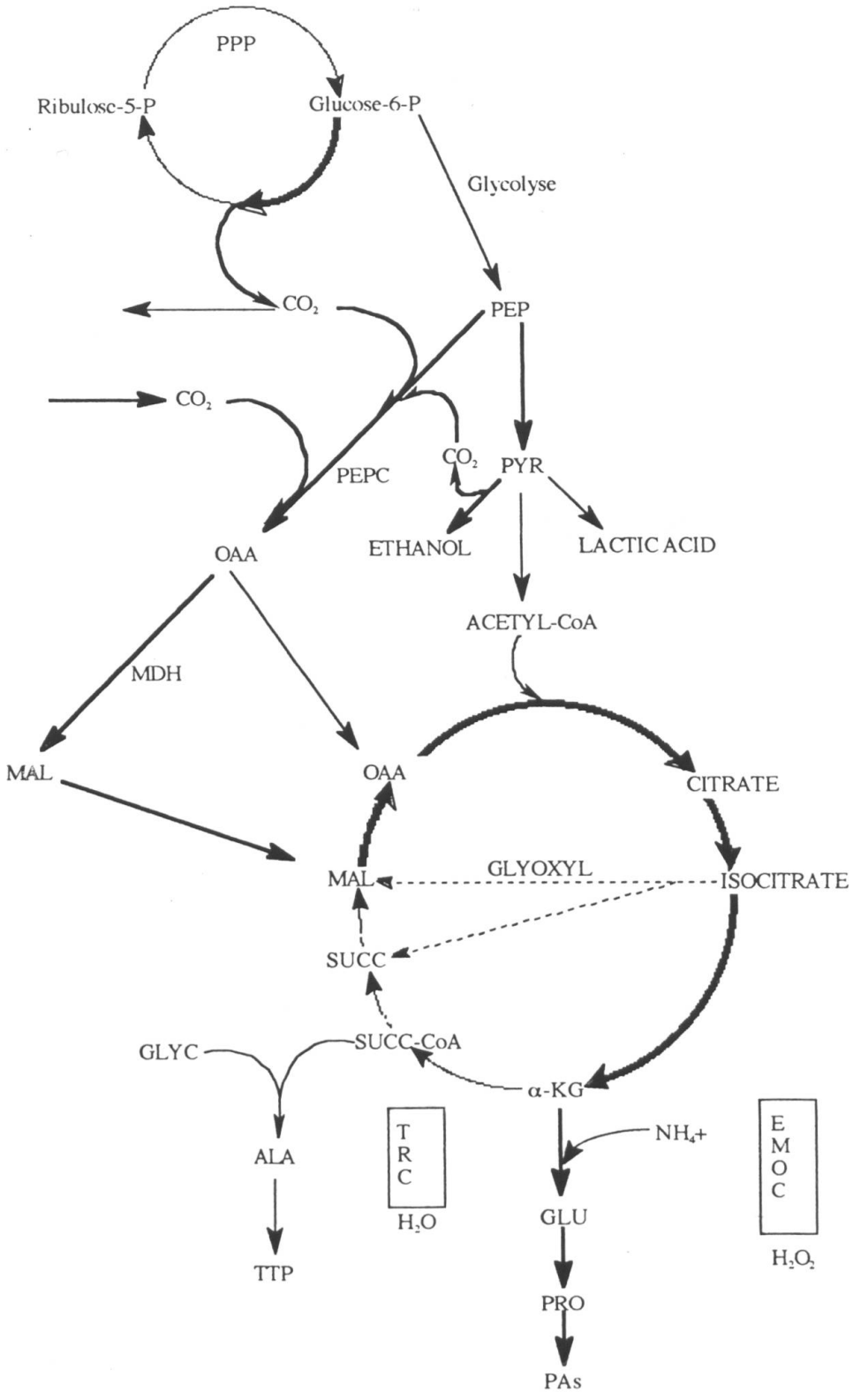

Fig. 3. — Tentative scheme showing the privileged glucose respiratory pathway of the HNO cells (full black lines). EMOC: extra mitochondrial oxidative pathway. TRC: terminal respiratory chain. PPP: pentose phosphate pathway. The glyoxylate cycle (interrupted lines) still has not been evaluated.

A larger heterotrophy of the HNO callus thus was demonstrated which was expected since it was shown to be unable of any photosynthetic activity (BISBIS & al., 1994b). The 20% auxotrophy necessarily account for non-photosynthetic CO_2 fixation. Phospho-enolpyruvate (PEP) aided by phosphoenolpyruvate carboxylase (PEPC) has been shown to be a nonphotosynthetic way of carbon fixation particularly in growing plant cultures (DE RIEK & al., 1991). Such a mechanism might be operating here, also in the N callus which still fixes CO_2 under darkness (BISBIS & al., 1994a). Hypothesizing that this PEPC-CO_2 fixation pathway is the main one outside photosynthesis, it can be considered that it is operating to a larger extent in HNO callus than in N callus, as seen from the comparable $^{14}CO_2$ dark fixation (BISBIS & al., 1994a) for unequal fresh and dry matter masses. PEPC-CO_2 fixation leads to malate accumulation. The larger PEPC-CO_2 fixation in the HNO callus thus might account for the higher malate dehydrogenase activities (see above) and the higher NAD/NADH dependent redox activities found in this callus, compared to the N one (CARRIÉ & al., 1994). This PEPC-CO_2 fixation might also account for the disturbed nitrogen metabolism with the accumulation of glutamate, proline, and polyamines in the HNO callus (LE DILY & al., 1993). Amino acid synthesis from Krebs cycle intermediates indeed requires replenishment of these intermediates. The most common reaction serving this anaplerotic function is the carboxylation of PEP which supplies oxaloacetic and malic acids (TURPIN & WEGER, 1990). A favoured pentose phosphate pathway (PPP) in the HNO callus (BISBIS & al., 1993) is not an objection to the above hypothesis, on the contrary. Following EMES & FOWLER (1979) indeed, the NADH needed for the reduction of nitrate to nitrite in non-photosynthetic plant cells could be provided through the triose-phosphate dehydrogenase reaction of glycolysis or through the PPP pathway. PLUMB-DHINDSA & al. (1979) already associated PPP and malate accumulation to provide NADPH for reductive biosynthesis.

The HNO cancerous cells thus should be characterized by several deviations of sugar metabolism as tentatively illustrated in Figure 3:

— the previously shown favoured PPP (BISBIS & al., 1993),

— the here suggested fermentation pathways

— the here indirectly confirmed bypass of the Krebs cycle through malate replenishment via PEP carbon fixation.

The latter bypass well justifies both the previously shown accumulation of nitrogenous compounds (LE DILY & al., 1993) and the deficiency in tetrapyrrole-containing compounds (HAGÈGE & al., 1992b).

Quinone generation in the early appearance of necrosis zones in the HNO callus (GASPAR & al., 1991) on the other hand might reflect the peculiar functioning of an extra-mitochondrial oxidative chain in these mitochondria and cytochrome deficient HNO cells. The poor differentiation capacities of the HNO cells finally are well explained through their higher reducing level.

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