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The Crabtree effect: a new look at the old problem*

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Inhibition of respiration by glucose, known as the Crabtree effect, has been observed in several tumours and some other highly glycolytic cells and tissues. Among mechanisms proposed to explain this effect were: competition between glycolysis and respiration for ADP or for inorganic phosphate, change of intracellular pH, change in the permeability of mitochondrial membranes, specific regulatory behaviour of glycolytic enzymes, and specific enzyme topography within the cell. None of these proposals alone seems satisfactory. The present article describes the research carried out in the author's laboratory, pointing to the role of Ca^{2+} in the mechanism of the Crabtree effect. This supposition is based on the following observations: (1) in Ehrlich ascites tumour cells glucose elicits a steady increase of the cytoplasmic concentration of free Ca^{2+}; (2) isolated Ehrlich ascites mitochondria and mitochondria within digitonin-permeabilised cells, preloaded with Ca^{2+}, exhibit a depression of State 3 respiration and lowering of the rate of ATP synthesis; (3) ATPase activity of toluene-permeabilised Ehrlich ascites mitochondria becomes substantially inhibited at micromolar concentrations of Ca^{2+}; (4) Ca^{2+} potentiates the effect of the inhibitory subunit of F_1F_o-ATPase. These results allow to hypothesize on the following sequence of events: (1) glucose elevates the cytoplasmic concentration of Ca^{2+}; (2) this elicits an increased accumulation of Ca^{2+} in mitochondria; (3) loading of mitochondria with Ca^{2+} leads to an increased association of the inhibitory subunit with F_1F_o which results in (4) the inhibition of coupled respiration. The importance of these mechanisms for glycolytic and rapidly proliferating cells is discussed.

A peculiar response of malignant tissues to glucose was first observed by Herbert G. Crabtree [1] in 1929 and since then is known under his name as the "Crabtree effect". He found that, in contrast to normal tissues like liver and kidney where glucose slightly increased the respiration of tissue slices or was without effect, the rate of oxygen uptake by sarcomas, carcinomas and other malignant cells and tissues was inhibited by some 10%. This effect was confirmed by other authors [2–8] who observed even a higher inhibition, in some cases exceeding 50% (for an early review see [9]). It has been subsequently shown that the Crabtree effect is not a peculiarity of malignant tissues, as it has also been observed in some normal tissues like spermatozoa [10, 11], proliferating thymocytes [12], intestinal mucosa [13] and, interestingly, mammalian embryos at their very early developmental stage [14]. A common feature of all these tissues, both malignant and normal, is a high proliferation rate and/or high glycolysis.

Application of the polarographic method for studying cellular respiration revealed that in-

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hibition of respiration following glucose addition was often preceded by a short-lasting increase of oxygen uptake [15]. A typical picture is illustrated in Fig. 1. The increase of O_2 uptake immediately following addition of glucose could be explained by rapid phosphorylation of this hexose inside the cell, resulting in an increase of cellular ADP concentration and a shift towards the active state ("state 3") respiration [16, 17]. However, the nature of the inhibition of oxygen uptake which followed thereafter and was more or less permanent has been a subject of numerous speculations.

First attempts to explain its mechanism pointed to the competition between respiration and glycolysis for precursors of ATP production, in particular for ADP [16, 17] and inorganic phosphate [8, 18, 19]. However, this explanation is not satisfactory since deoxyglucose, which is phosphorylated in the cytoplasm but is not further metabolized and therefore does not generate ATP, produced a similar, or even higher, inhibition of respiration as did glucose [6] and since phosphate transport into the cell has been shown to be fast enough [6]. Other attempts to explain the mechanisms of the Crabtree effect included a shift of intracellular pH [9], a change of permeability properties of the inner mitochondrial membrane [20], specific isoenzyme pattern of the glycolytic pathway and regulatory behaviour of key enzymes of this pathway [21], and specific topography of enzymes in rapidly growing tumours, in particular of membrane-bound hexokinase [22]. However, none of these proposals provides a fully satisfactory explanation of how glucose (and deoxyglucose) may inhibit oxygen consumption in some tissues.

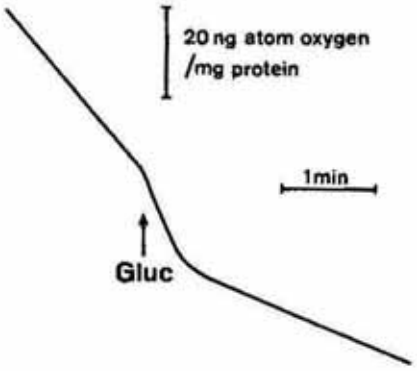

Fig. 1. Effect of glucose on the respiration of Ehrlich ascites tumour cells.
The incubation medium contained 140 mM NaCl, 3 mM KCl, 0.6 mM $MgCl_2$, 2 mM $CaCl_2$, 1.5 mM phosphate and 10 mM Hepes (Na salt), pH 7.6. Glucose (Gluc) was added to 3 mM final concentration. The temperature was 30°C.

This article summarizes our studies, carried out during the last few years, which shed a new light on the mechanism of the Crabtree effect involving a possible role of intracellular Ca^{2+}. Using the intracellular fluorescent calcium probe, Fura-2, we observed an increase of free calcium ion concentration in the cytoplasm shortly after supplementing the incubation medium with glucose or deoxyglucose [23–25]. This increase was observed in both cell suspensions (Fig. 2) and in single cells using the video imaging system (Fig. 3). As clearly shown in Fig. 2, the increased level of cytoplasmic Ca^{2+} was observed independently of whether the external medium contained Ca^{2+} or was nominally Ca^{2+}-free (i.e. in the presence of 2 mM EGTA), although it was higher in Ca^{2+}-containing media. Therefore, it can be concluded that this increase of cytoplasmic Ca^{2+} concentration

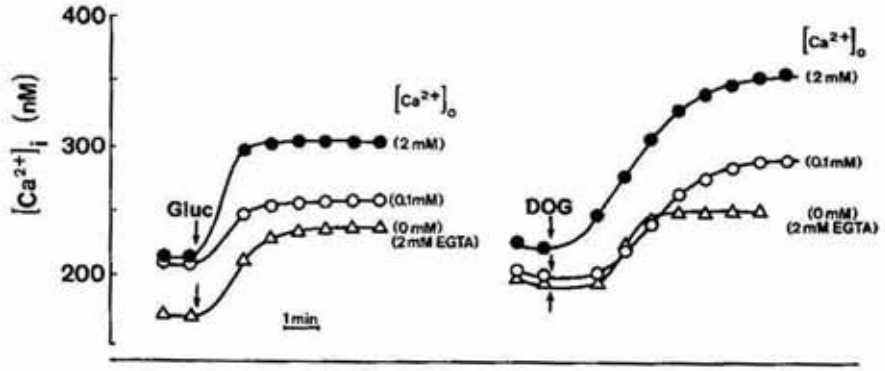

Fig. 2. Effect of glucose and deoxyglucose on cytoplasmic Ca^{2+} in Ehrlich ascites tumour cells.
The concentration of cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) was measured by Fura-2 fluorescence at various Ca^{2+} concentrations in the medium ($[Ca^{2+}]_o$). Glucose (Gluc) and deoxyglucose (DOG) were added to 10 mM final concentration. From [23] with permission.

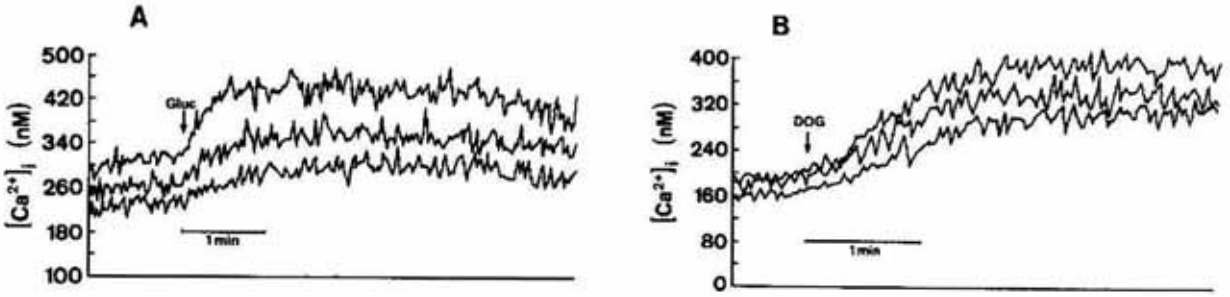

Fig. 3. Effect of glucose (A) and deoxyglucose (B) on cytoplasmic Ca^{2+} concentration in single Ehrlich ascites tumour cells.
Each panel shows traces for three individual cells. The incubation medium contained 0.1 mM $CaCl_2$. Other conditions were as in Fig. 2. Gluc, glucose; DOG, deoxyglucose. From [24] with permission.

occurred primarily at the expense of intracellular Ca^{2+} stores. It was also shown [23–25] that depletion of the endoplasmic reticulum calcium stores by thapsigargin, a specific inhibitor of Ca^{2+}-pumping ATPase [26], substantially diminished the Ca^{2+} increase which followed the addition of glucose or deoxyglucose (Fig. 4).

A further support to the concept that increased cytoplasmic concentration of free Ca^{2+} is responsible for the Crabtree effect in Ehrlich ascites tumour cells was provided by the following observations (Wojtczak *et al.*, unpublished). Firstly, the inhibition of O2 uptake following glucose addition was significantly reduced when the cells were pre-loaded with the calcium chelator, 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA). Secondly, the Crabtree effect was enhanced in parallel with the replenishment of intracellular stores with Ca^{2+} by preincubation of freshly isolated cells for various periods of time in Ca^{2+}-containing media.

Increased Ca^{2+} concentration in the cytoplasm may have several important consequences for the cell metabolism. Since cell respiration mainly depends on the status of mitochondria, we focused our attention on the response of Ehrlich ascites mitochondria to elevated Ca^{2+} concentration. It appeared [27] that a single pulse of micromolar Ca^{2+} resulted in a decrease of ADP phosphorylation and of the uncoupler-stimulated ATPase activity in isolated Ehrlich ascites tumour mitochondria (Fig. 5, compare traces A and B). It also decreased the response of the mitochondrial membrane potential to ADP addition (Fig. 5, traces C and D). The dependence of the degree of inhibition on the amount of Ca^{2+} added is shown in Fig. 6. It has to be stressed that under these conditions Ca^{2+} was rapidly taken up by mitochondria as illustrated by a rapid return of the membrane potential to its steady-state high value (Fig. 5, traces C and D).

Similar effects could be observed when mitochondria (in this case in permeabilised Ehrlich ascites tumour cells), instead of being exposed to a single pulse of micromolar Ca^{2+}, were subjected in a prolonged way to submicromolar calcium concentrations [27]. In that case all important parameters of energy coupling, like oxygen uptake in state 3 (but not in state 4), respiratory control, ATP synthesis and the response of the membrane potential to ADP, became dramatically affected at Ca^{2+} concen-

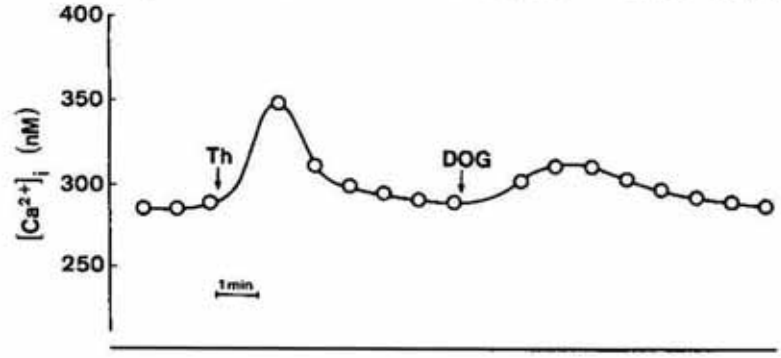

Fig. 4. Effect of thapsigargin on deoxyglucose-induced increase of cytoplasmic Ca^{2+} concentration in Ehrlich ascites tumour cells.
The medium contained 0.1 mM $CaCl_2$. Thapsigargin (Th) was added to 0.25 μM final concentration and deoxyglucose (DOG) to 10 mM. From [23] with permission.

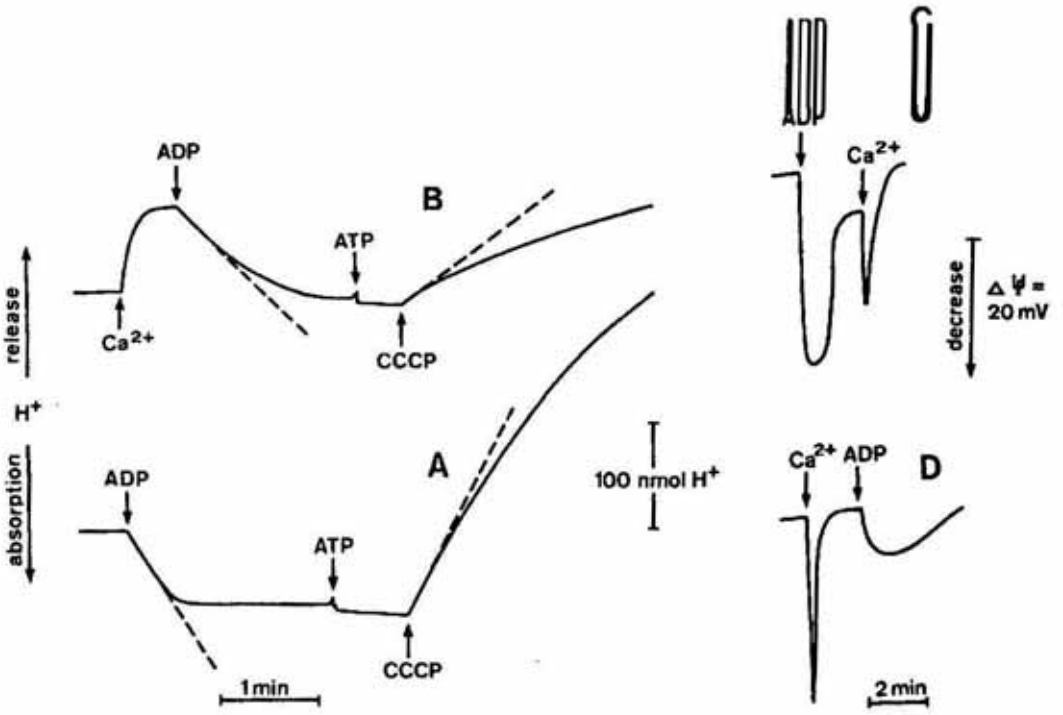

Fig. 5. Effect of Ca^{2+} on ATP synthesis and hydrolysis and on changes of the membrane potential upon state 4/state 3 transition in Ehrlich ascites tumour mitochondria.
Traces A and B illustrate ADP phosphorylation and ATP hydrolysis in isolated mitochondria measured as alkalisation and acidification, respectively, of the medium. Traces C and D show changes of the membrane potential ($\Delta\Psi$), measured with a tetraphenylphosphonium electrode, in mitochondria *"in situ"*, i.e., in digitonin-permeabilised cells. The amount of added $CaCl_2$, where indicated by the arrows, was 50 nmol/mg mitochondrial protein. Numbers at the traces indicate the rate of H^+ (in nmol/min per mg protein) taken up (downward deflection) or liberated (upward deflection). CCCP, carbonyl cyanide *m*-chlorophenylhydrazone. From [27 and 28] with permission**, combined and modified.

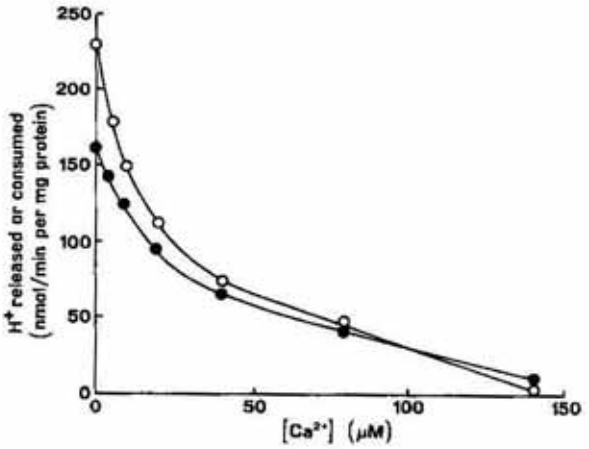

Fig. 6. Dependence of the rates of ADP phosphorylation and ATP hydrolysis in Ehrlich ascites tumour mitochondria on Ca^{2+} concentration.
The reactions were followed, as in Fig. 5, by measuring the uptake or the release of H^+ during ADP phosphorylation (●) or ATP hydrolysis (○), respectively. The amount of mitochondria corresponded to 1 mg/ml and hence the numbers at the abscissa correspond to nmol Ca^{2+}/mg protein. From [28] with permission**.

tration of about 0.3 μM (Fig. 7). It has to be noted that glucose and deoxyglucose elicited in intact cells an increase of Ca^{2+} concentration above the level of 0.3 μM (Figs. 2 and 3).

All these results clearly point to a lesion in either the coupling machinery or ATP/ADP translocation across the inner mitochondrial membrane. The former possibility was substantiated by the experiment in which the Ca^{2+} effect on mitochondrial ATPase activity (i.e. the reversal of ATP synthesis catalysed by the same enzyme system, the F_1F_o complex) was compared in intact and permeabilised mitochondria. It was shown [28] that hydrolysis of ATP was also inhibited in toluene-permeabilised mitochondria (Fig. 8), thus indicating that the permeability factor was not involved and that excessive loading of Ehrlich ascites tumour mitochondria with Ca^{2+} may affect functioning of the ATP synthesizing assembly.

One of possible mechanisms of this effect might be a reversible association/dissociation of the natural inhibitory subunit, the Pullman-Monroy inhibitor. To check this possibility, the effect of Ca^{2+} on the ATPase activity in submitochondrial "inside-out" particles prepared from Ehrlich ascites tumour mitochondria was

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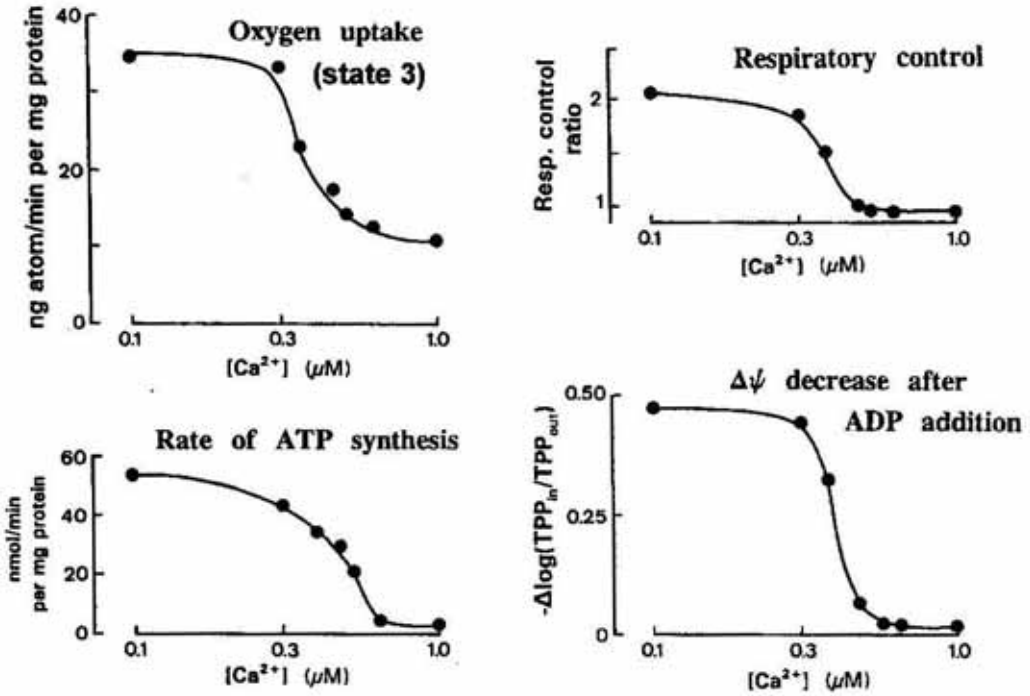

Fig. 7. Dependence of the respiration, the respiratory control, the rate of ATP synthesis and the decrease of the mitochondrial membrane potential upon ADP addition in permeabilised Ehrlich ascites tumour cells incubated at submicromolar Ca^{2+} concentrations.
From [27] with permission.

examined [28]. It was found that this activity was strongly inhibited by micromolar concentrations of Ca^{2+} (Fig. 9), half inhibition being attained at about 15 μM Ca^{2+}. However, when the particles were depleted of the inhibitory subunit by alkali treatment and Sephadex filtration, ATPase activity became insensitive to a Ca^{2+} concentration as high as 100 μM. Supplementation of the particles with the inhibitory subunit isolated from beef heart mitochondria restored the original high sensitivity towards Ca^{2+} (Fig. 9). This experiment convincingly shows that functional re-association of the inhibitory subunit with the F_1F_o complex in Ehrlich ascites tumour mitochondria makes it highly sensitive to calcium ions. It has to be mentioned that ATPase activity of rat liver mitochondria was also inhibited by Ca^{2+}. However, in submitochondrial particles from these mitochondria comparable inhibition was obtained with Ca^{2+} concentrations ten times higher than in the case of particles from tumour mitochondria.

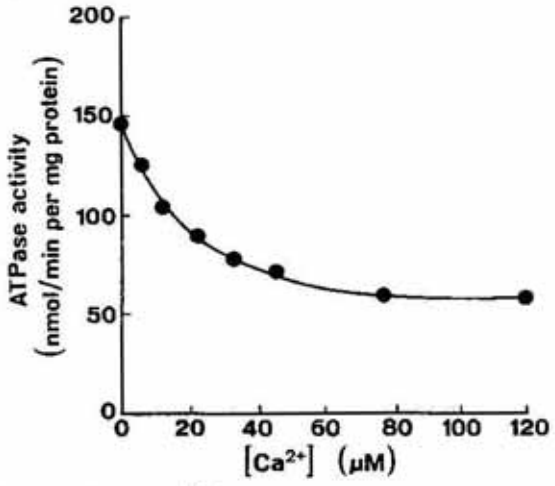

Fig. 8. Effect of Ca^{2+} on ATPase activity in toluene-permeabilised Ehrlich ascites tumour mitochondria.
From [28] with permission**.

On the basis of these results we can propose the following sequence of events leading to a partial inhibition of cell respiration following supplementation of the medium with glucose [28]. Firstly, glucose gives rise to a release of calcium from its intracellular stores, presumably the endoplasmic reticulum. Secondly, the increased Ca^{2+} concentration in the cytoplasm results in an increased accumulation of these ions in mitochondria. And this leads, in turn, to the association of the inhibitory subunit with the ATP synthase complex, thus inhibiting ADP phosphorylation and, consequently, the coupled respiration.

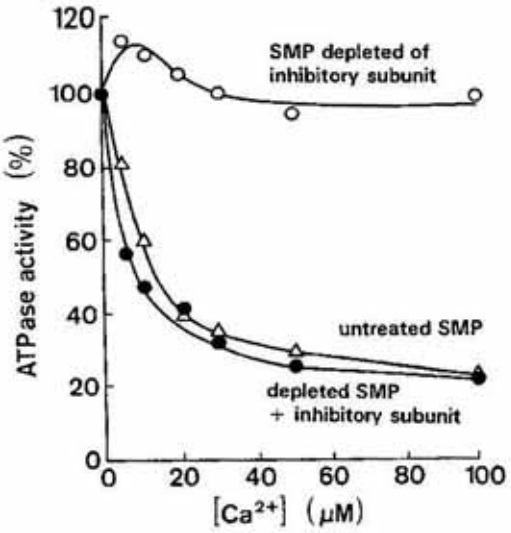

Fig. 9. Effect of Ca^{2+} on ATPase activity in submitochondrial particles (SMP) from Ehrlich ascites tumour.

ATPase was measured spectrophotometrically in the ATP-regenerating system. From [28] with permission**.

Although this scheme is supported by the available experimental data, a number of points remain to be answered. Firstly, the mechanism of the hexose-induced increase of cytoplasmic Ca^{2+} has to be clarified. It seems likely that deoxyglucose may inhibit active removal of Ca^{2+} from the cytoplasm due to a rapid depletion of cytoplasmic ATP [29]. However, such an explanation does not hold for glucose which, on the contrary, can only increase ATP production (except, occasionally, for a very short period immediately after glucose supplementation [17, 30]). The second important point to be elucidated is the difference in susceptibility to Ca^{2+} of the association between the inhibitory subunit and the F_1F_o complex in normal and malignant or highly glycolytic tissues. It can be thought that this difference may be related to differences in the phospholipid environment or different properties of F_1F_o complexes.

Finally, it has to be stressed that in most mammalian cells the increased concentration of cytoplasmic Ca^{2+} and the resulting increase of the level of these ions in the mitochondrial matrix elicit an increase, and not a decrease, of cellular respiration mainly as a result of activation of several dehydrogenases [31]. A strikingly different response to elevated cytoplasmic Ca^{2+} concentration by highly glycolytic tissues is therefore intriguing and raises the question of possible biological importance of the Crabtree effect. One can speculate that one of the advantages of switching from respiration to anaerobic energy production, which occurs in glycolytic cells under conditions of plentiful glucose supply, is the protection of the cell from deleterious effects of oxygen free radicals generated as "by-products" of the aerobic energy metabolism.

Recently, we have obtained indications that the Crabtree effect may also affect phospholipid metabolism. The so called base exchange reaction occurs in the endoplasmic reticulum and is the only pathway of phosphatidylserine synthesis in animal cells (for review see [32]). This process requires millimolar concentrations of Ca^{2+}, and it has been recently demonstrated [33, 34] that this reaction proceeds on the luminal side of the membrane or, at least, requires a high concentration of Ca^{2+} inside the endoplasmic reticulum cisterns. As a result, depletion of intracellular Ca^{2+} stores in glioma cells, e.g. by thapsigargin, resulted in an inhibition of phosphatidylserine synthesis [35]. We found [36] that in Ehrlich ascites tumour cells synthesis of phosphatidylserine was inhibited by glucose and deoxyglucose, though to a smaller degree than by thapsigargin (Table 1). This observation confirms the previously formulated conclusion that glucose and deoxyglucose release Ca^{2+} from the endoplasmic reticulum stores. Since phosphatidylserine is a well known activator of protein kinase C [37], decreased formation of this phospholipid in the presence of glucose may have far reaching metabolic consequences.

Table 1

Effect of glucose, deoxyglucose and thapsigargin on the incorporation of [^{14}C]serine into phospholipids.

[^{14}C]Serine incorporation in the control (without additions) amounted to 95 ± 27 (6) pmol/15 min per 50 million cells. All values are means ±SD for the number of experiments indicated in parentheses. From [36] with permission.

Addition	[^{14}C]Serine incorporation (% of the control)
Glucose (10 mM)	69 ± 14 (6)
Deoxyglucose (10 mM)	42 ± 7 (6)
Thapsigargin (0.2 μM)	18 ± 4 (3)

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