KINETICS OF OXYGEN CONSUMPTION AND LUMINESCENCE OF PYRIDINE NUCLEOTIDES AND CYANINE DYE 3',3'-DIETHYLTHIODICARBOCYANINE IODIDE AFTER ENERGIZATION OF EHRLICH'S ASCITES CARCINOMA CELLS BY GLUCOSE

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The widespread use of some of the cyanine dyes, especially 3'3'-diethylthiodicarbocyanide iodide [dis-C_2-(5)], as indicators of transmembrane potentials (TMP) of cells [10], mitochondria [1], sarcoplasmic reticulum [2], and liposomes [16], is due to the sufficiently high rate of response to changes of potential and to the high amplitude of changes of the signal. Hyperpolarization of the membranes of these objects leads to accumulation of molecules of the dye in the membrane and to quenching of luminescence, whereas depolarization releases the dye [1, 16].

One of the disadvantages of these cyanine dyes is that, even in low concentrations (2×10^{-8} M) they inhibit the mitochondrial respiratory chain. Unlike with rotenone the degree of inhibition of mitochondria by the dye depends on the level of their TMP. At low potentials and in the presence of an uncoupler, the dye thus leaves the membrane and inhibition is abolished [11].

Experiments on Ehrlich's ascites carcinoma (EAC) cells have shown that cyanine dyes inhibit their endogenous respiration [13]. Hence, if dis-C_2-(5) penetrates into the cell and interacts with mitochondrial membranes, this raises the question of what contribution the mitochondria make to changes in luminescence of dis-C_2-(5) and whether this can be used to study changes in mitochondrial TMP *in vivo*.

Addition of glucose to a suspension of EAC cells often causes a sharp decrease in their ATP concentration [8, 14]. One hypothesis explains this phenomenon by inhibition of glycolysis by a high NADH level [8], for the absence of glycerophosphate shuttle mechanisms in these cells does not allow the mitochondria to oxidize cytoplasmic NADH rapidly enough.

Measurements of luminescence of NADH and dis-C_2-(5), the pH of the medium, and oxygen consumption, undertaken in the investigation described below, allow these mechanisms of regulation of glycolysis and of the overall energy metabolism of tumor cells to be examined.

EXPERIMENTAL METHOD

Mouse EAC cells were isolated on the 7th-8th day after transplantation, washed twice in Ca^{2+}-free Ringer's solution at pH 7.4, then resuspended in 10 ml of incubation medium of the following composition: 150 mM NaCl, 6 mM KCl, 10 mM HEPES, pH 7.4. The cell suspension was transferred in a volume of 0.2-0.4 ml into a measuring cell containing 2 ml of the same medium. Measurements were made at 37°C with mixing. The design of the cell was such that the intensity of the luminescence of pyridine nucleotides (PN) and of dis-C_2-(5), the oxygen concentration, and the pH of the medium could be measured simultaneously [3]. Luminescence of PN was excited at 365 nm and recorded at 465 nm; luminescence of dis-C_2-(5) was excited at 579 nm and recorded at 670 nm. The pH was measured by an ion-selective electrode and changes in the oxygen concentration in the cell by means of a closed electrode of the Clark type [5]. The fluorescent dye dis-C_2-(5) was supplied by Dr. T. T. B. Simons from

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King's College, London. Valinomycin was obtained from Serva (West Germany) and oligomycin and rotenone from Sigma (USA).

EXPERIMENTAL RESULTS

The rate of oxygen consumption, changes in pH of the medium, and reduction of PN on addition of the EAC cells to the incubation medium are illustrated in Fig. 1. In the absence of exogenous substrates no changes in pH of the medium were observed. The level of reduced PN was quite high when the EAC cells were introduced into the measuring cell (on account of the hypoxic conditions of keeping), but fell sharply in the course of 1-2 min. This indicates their oxidation and the fact that under these conditions the mitochondria were in an active state, controlled by oxidative phosphorylation, for oligomycin induces reduction of PN and inhibits respiration (by about 75%). Addition of FCCP causes oxidation of PN. This indicates that changes in the intensity of luminescence reflect oxidation–reduction transitions of mitochondrial PN.

On addition of the EAC cells to the measuring cell a high velocity of oxygen consumption was observed, which was blocked by rotenone and by antimycin A, and also inhibited to different degrees by oligomycin and glucose, and stimulated by the uncoupler FCCP* (Fig. 1). Exhaustion of oxygen in the measuring cell again reduced PN. Addition of glucose to such cells led to marked acidification of the medium through activation of glycolysis and incompletely reversible reduction of cytoplasmic NAD^+.

It can thus be concluded that in the preparation used the cells were in a somewhat deenergized state (with a high ADP/ATP ratio). Luminescence of PN was due both to mitochondrial PN and to cytoplasmic NADH (in the case of activation by glucose).

On the addition of glucose to the cell suspension in the absence of inhibitors of the respiratory chain and of oxidative phosphorylation reduction of PN, inhibition of respiration by about 60%, and acidification of the medium were observed to take place rapidly (Fig. 2). These changes were evidently connected with activation of glycolysis and the appearance of lactate in the incubation medium [15]. PN, reduced on the addition of glucose, were not oxidized on the addition of FCCP, indicating their cytoplasmic origin. The effect of a high rate of glycolysis under conditions of a full oxygen supply, which is known to be characteristic of tumor cells, was thus observed in this case. The reason was that in such cells the glycerophosphate shuttle mechanism does not function actively because of glycerophosphate dehydrogenase deficiency [4, 6]. Although unable to oxidize cytoplasmic NADH by the mitochondrial pathway, the tumor cells can oxidize it by the lactate dehydrogenase reaction. Under those circumstances, lactate accumulates under aerobic conditions [15].

The rate of acidification of the medium decreased and PN were partially oxidized 2-3 min after the addition of glucose. Inhibition of glycolysis was evidently due, on the one hand, to the rise in the ATP concentration on account of mitochondrial energy production, and on the other hand to inhibition of the initial stages of glycolysis by a high NADH level [8, 17]. Later, against the background of a low rate of respiration and acidification, a phase of slow reduction of PN was observed; this evidently reflects reduction of mitochondrial PN, for on the addition of FCCP this fraction of PN was oxidized, but the addition of rotenone at different points of this phase led to rapid reduction of PN: the sooner the addition was made, the more rapid its reduction (Fig. 2). Addition of rotenone against the background of glucose also led to inhibition of respiration and to an increase in the rate of acidification of the medium. Whereas before the additions of rotenone the cells were able to oxidize pyruvate in the mitochondria, they now transformed it into lactate, thus exhibiting competition of the pyruvate dehydrogenase system of the mitochondria and LDH for the common substrate and the regulatory role of pyruvate in glycolytic function under those conditions. The added FCCP oxidized mitochondrial PN to a greater degree than it reduced rotenone or dis-C_2-(5), thereby showing that part of the cytoplasmic NADH is oxidized after the addition of rotenone. Consequently, the increase in the rate of acidification of the medium can be ascribed not only to the fact that the part of the pyruvate that was previously utilized for respiration by the mitochondria was not utilized for lactic acid formation, but also to the increase in the rate of glycolysis on account of oxidation of cytoplasmic NADH during reduction of pyruvate.

*Carbonyl cyanide p-fluoromethoxyphenylhydrazone.

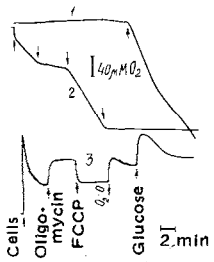

Fig. 1

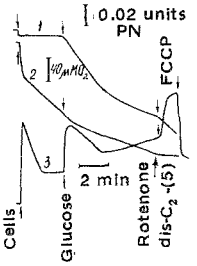

Fig. 2

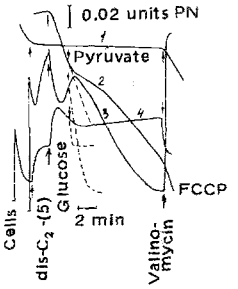

Fig. 3

Fig. 1. Action of oligomycin (1 μg/ml), FCCP (2×10^{-6} M), and glucose (5 mM) on EAC (1.6×10^{7} cells/ml): 1) change in pH of medium; 2) oxygen consumption; 3) change in fluorescence of PN.

Fig. 2. Action of glucose (5 mM), rotenone (1 μg/ml), and FCCP on EAC cells. Legend as to Fig. 1.

Fig. 3. Action of glucose (5 mM), pyruvate (5 mM), FCCP, and valinomycin (1 μg/ml) on EAC cells in the presence of 2×10^{-6} M dis-C_2-(5). 1) Oxygen consumption; 2) change in pH of medium; 3) change in fluorescene of dis-C_2-(5); 4) change in fluorescence of PN. Broken line shows change in reaction kinetics after addition of 5 mM pyruvate.

The action of glucose on EAC cells in the presence of dis-C_2-(5) is shown in Fig. 3. Addition of 2×10^{-6} M dis-C_2-(5) to the cell suspension was shown to lead to inhibition of respiration and to reduction of PN, just as in the parallel experiments with rotenone. Responses of PN and PH to glucose also were analogous in the presence of rotenone and dis-C_2-(5). Hence, if dis-C_2-(5) penetrates into the cell and interacts with mitochondrial membranes, the following questions arise: what is the contribution of the mitochondria to changes in luminescence of dis-C_2-(5) and can this contribution be used in order to study changes in mitochondrial TMP inside intact cells.

Changes in luminescence of dis-C_2-(5) in the course of energization of the cells by glucose are shown in Fig. 3. After addition of dis-C_2-(5) to the cell suspension, an increase in the intensity of luminescence was observed (a decrease in TMP). The rate of increase of luminescence was proportional to the dis-C_2-(5) concentration and correlated with the rate of reduction of PN and the degree of inhibition of respiration. In the presence of low concentrations of dis-C_2-(5) the process was accelerated by the addition of rotenone. Changes in luminescence of dis-C_2-(5) in the presence of respiratory chain inhibitors were sensitive to oligomycin.

Addition of glucose led to a decrease in luminescence with a complex kinetics. The rapid phase of growth of the potential was replaced by a phase of decline. This period corresponded to the period of inhibition of primary activation of glycolysis. A slow decrease in the intensity of luminescence was then observed, down to a very low level, which ought to correspond to the appearance of a TMP of considerable magnitude. This period coincided with that of slow acidification of the medium. After the luminescence of dis-C_2-(5) reached a minimal level it remained constant.

The potential arising on account of energization of the cells by glucose was dissipated by addition of FCCP, valinomycin, and oligomycin by 90% of that due to FCCP alone, but was not affected by antimycin A.

It can thus be concluded that in the absence of inhibitors of mitochondrial oxidative phosphorylation luminescence of dis-C_2-(5) reflects mainly mitochondrial TMP arising through H^+-ATPase activity, and not the cytoplasmic membrane potential.

In the presence of oligomycin and antimycin A changes in the intensity of luminescence of dis-C_2-(5) in response to glucose did not exceed 10% of the changes observed in the absence of inhibitors. These changes can be ascribed to changes in the cytoplasmic membrane potential. They are sensitive to ouabain and depend on the external K^+ concentration in the presence of valinomycin, both in the present experiments and in [12].

It was shown previously [13] that unlike rotenone, dis-C_2-(5) weakly inhibits NAD-dependent respiration of isolated mitochondria in the presence of uncoupler. This is evidently due to outflow of the dye from the mitochondria when membrane potentials are low. In the present investigation a similar effect of FCCP and valinomycin was observed, for they not only dissipated the potential but also activated respiration and oxidized PN (Fig. 3). This effect was not observed in the presence of rotenone, just as on isolated mitochondria, although the TMP also fell strongly.

It can thus be concluded that energization of EAC cells by glucose in the presence of rotenone or dis-C_2-(5) leads to fluctuating changes of TMP on the mitochondrial membranes (rapid growth at first, followed by a fall and slow growth) on account of H^+-ATPase activity, utilizing glycolytic ATP.

The similar kinetics of the changes in pH and PN in the absence of rotenone indicate that similar reactions took place in this case also.

Despite the presence of an inhibitory effect, dis-C_2-(5) and other cyanine dyes of this series can thus be used to record mitochondrial TMP continuously in EAC cells. Changes in potential on the cytoplasmic membrane can be followed only in the presence of inhibitors of the respiratory chain and of oxidative phosphorylation.

Since in the presence of rotenone or antimycin A mitochondria are supplied chiefly with glycolytic ATP, the kinetics of the changes in mitochondrial TMP can be regarded as a kinetics reflecting changes in the intracellular ATP level. This complex kinetics of the changes in potential can be explained as follows on the basis of data obtained in [8, 14] regarding changes in the ATP content and the total intracellular reserve of adenine nucleotides on the addition of glucose. The first phase of growth of potential is evidently connected with an increase in the ATP concentration in glycolytic reactions on account of oxidation of NADH by endogenous acceptors (endogenous reserves of pyruvate and α-ketoglutarate). The increase in the NADH concentration blocks energy-producing reactions of glycolysis at the glyceraldehyde-3-phosphate dehydrogenase level, leading to a fall in the ATP level on account of its utilization in the preceding reactions [8, 14], and also on account of an overall decrease in the reserves of adenine nucleotides because of activation of AMP deaminase [8]. Later, in the presence of a low ATP concentration and accumulation of fructose-1,6-diphosphate optimal conditions are created for activation of pyruvate kinase [8]; this leads to an increase in the endogenous pyruvate concentration and enables oxidation of cytoplasmic NADH, so that the energy-yielding reactions of glycolysis can take place normally.

Addition of endogenous pyruvate is known to prevent the fall in the ATP content and accumulation of fructose-1,6-diphosphate in response to addition of glucose to EAC cells [8]. Figure 3 shows that addition of pyruvate (5 mM) in the presence of glucose leads to oxidation of NADH and to a rapid rise in mitochondrial TMP. Addition of pyruvate before glucose changes the response to glucose in such a way that the phases of the fall of potential and inhibition of acidification of the medium disappear, and TMP on the mitochondrial membranes (and, consequently, the intracellular ATP level) reaches a maximum within 2-3 min.

These data thus confirm the view that the fall in the ATP content in EAC cells in response to addition of glucose is connected with inhibition of glycolysis by the high NADH level and the impossibility of oxidation of NADH by the mitochondria.

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