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THE REOXIDATION OF CYTOPLASMIC REDUCING EQUIVALENTS IN EHRLICH ASCITES TUMOR CELLS

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SUMMARY

1. Addition of glucose to ELD (Ehrlich–Lettre' hyperdiploid) ascites tumor cells inhibited by rotenone caused an oxidation–reduction cycle of cytochrome *b* and oxidation of cytochrome *c* but it did not affect the inhibition of the endogenous respiration.

2. The effect of substrates, inhibitors, and uncouplers of oxidative phosphorylation on the transition between the oxidized and the reduced steady state of cytochrome *b* induced by glucose in rotenone-inhibited cells was tested. The spectrophotometric data obtained support the hypothesis that this transition is determined by the initial lowering and the subsequent increase in the phosphate potential in the cytoplasmic compartment of the glycolyzing cell.

3. The reduction of cytochromes *b* and *c* induced by glucose in rotenone-inhibited cells in the presence of vitamin K_3 was shown to be biphasic. This reduction, like that produced by vitamin K_3 alone, was significantly increased by oligomycin or aurovertin, while the biphasicity of the response was no longer apparent with these inhibitors.

4. Pretreatment with pyruvate of rotenone-inhibited cells made it possible to show, upon addition of glucose, a rapid translocation ($t_{1/2}$ about 700 msec) of reducing equivalents to a cytochrome *b*-type pigment with small or undetectable changes in the steady-state level of cytochrome *c*. This reaction, which may be tentatively assigned to the microsomal cytochrome b_5, was not competitive with the transfer of reducing equivalents to the mitochondria in the presence of vitamin K_3.

5. It can be concluded that in the strain of ascites cells used no direct transport of reducing equivalents occurs under physiological conditions from the cytosol to the mitochondrial cytochromes. On the other hand a pathway for the extramitochondrial oxidation of the cytosolic reducing equivalents has been evidentiated.

INTRODUCTION

The extent to which single reactions involved in the reoxidation of cytoplasmic reducing equivalents (such as intramitochondrial oxidation, utilization of reducing power for biosynthetic processes, microsomal hydroxilation) contribute to the avail-

Abbreviation TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole

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ability of NAD^+ and $NADP^+$ in the cell is not known and may represent one of the factors regulating the rate of aerobic production of lactate in glycolyzing cells.

Intact mitochondria isolated from mammalian cells, including tumor mitochondria, do not oxidize added reduced nicotinamide nucleotides[1-3], presumably because of the permeability barriers to them. Consequently, "shuttle mechanisms" (*i.e.*, the cyclic oxidoreduction of substrate couples in cytosol and mitochondria) have been proposed to account for the intramitochondrial oxidation of reducing equivalents[4-6].

The high rate of aerobic lactate production in tumor cells has been attributed to the lack of intracellular processes for the utilization of reducing equivalents formed in the extramitochondrial compartments, and particularly to a failure of their intramitochondrial oxidation[2,7,8]. This conclusion arises from the observation that the activity of enzymes involved in proposed mechanisms for the shuttling of hydrogen between cytosol and mitochondria is lacking, or very low, in different kinds of tumor cells. It has been shown, for instance, that Ehrlich ascites tumor cells are deficient in cytoplasmic α-glycerophosphate dehydrogenase (EC 1.1.1.8)[3,9] and β-hydroxybutyrate dehydrogenase (EC 1.1.1.30)[3] activities. Similar results have been reported for these enzymes in some solid tumors of rodents and human beings[2,10,11] (see ref. 12 for review).

However, the failure to find certain enzyme functions in the cytoplasmic and mitochondrial compartments of tumor cells does not allow final conclusions to be drawn. Such findings may be not generally applicable to all kinds of tumor tissues nor to different strains of the same tumor. It has been shown, indeed, that some strains of ascites tumor cells can form α-glycerophosphate during anaerobic glycolysis[13] or in the presence of rotenone[14], and that α-glycerophosphate can be oxidized, as a flavin-linked substrate, both by isolated ascites mitochondria[3,13] and by the intact cells[14]. EMMELOT *et al.*[15] have also reported that some rat hepatomas produce appreciable quantities of α-glycerophosphate from glycolytic intermediates.

In earlier work, TERRANOVA and co-workers[16-18] have shown that the addition of glucose to hyperdiploid Ehrlich ascites tumor cells is able to release about 50 % of the inhibition of the endogenous respiration induced by rotenone. This effect of glucose has been attributed to the activity of a shuttle which transfers reducing equivalents to the respiratory chain beyond the rotenone-inhibited site.

In the attempt to look further into the problem of intracellular hydrogen transport in ascites carcinoma cells we have undertaken the present investigation in the Ehrlich–Lettre' strain of these cells. This study was prompted by the possibility of following directly, with spectrophotometric and fluorimetric techniques, the redox changes of electron carriers in correlation with the feeding of reducing equivalents generated during the glycolysis.

With the strain of cells used in the present work, it was impossible to find any stimulation by glucose of the respiration blocked by rotenone. Nevertheless changes in the steady-state level of respiratory carriers were observed in these conditions, which are interpreted to indicate the operation of a rotenone-insensitive shuttle of rather low efficiency. In addition a transfer of reducing equivalents to a cytochrome *b*-type pigment was demonstrated, which was not competitive with this shuttle activated by vitamin K_3, and may be mainly microsomal in origin.

MATERIALS AND METHODS

ELD (Ehrlich–Lettre' hyperdiploid) ascites tumor cells were harvested 7–8 days after inoculation in albino mice (ICR Swiss). The cells were washed and freed from hemoglobin by one or two 60-sec differential lyses in distilled water, and then were suspended at the final concentration of 50–70 mg dry wt./ml in an isotonic saline medium (154 mM NaCl–6.2 mM KCl–11 mM sodium phosphate buffer (pH 7.4))[19]. Aliquots of this suspension were diluted with the same medium for each experiment.

O_2 uptake was measured polarographically with a Clark oxygen electrode. Room- and liquid nitrogen-temperature difference spectra were measured with a split-beam spectrophotometer[20,21]. Low-temperature spectra were determined with the trapped steady-state technique of CHANCE AND SPENCER[22]. Cytochromes *b* (430–410 mμ) and *c* (550–540 mμ) were measured separately with a double-beam spectrophotometer[23], or simultaneously with a double double-beam spectrophotometer[24]. Rapid-reaction kinetics were performed in the 20-ml regenerative flow apparatus[25]. Pyridine nucleotides reduction was measured with a fluorometer[26]. All the experiments were carried out at room temperature.

The 4,5,6,7-tetrachloro-2-trifluoromethylbenzaimidazole (TTFB) was kindly supplied by Dr. R. B. Beechey of Shell Research, Milstead Laboratory of Chemical Enzymology, Sittingbourne, Kent (Great Britain). Aurovertin was a generous gift of Prof. H. A. LARDY. All other chemicals were purchased from Sigma Chemical Co. except for sodium pyruvate (Eastman Organic Chemicals), NaF and Na_2S (J. T. Baker Chemical Co.).

RESULTS

Lack of glucose effect on rotenone-inhibited respiration

TERRANOVA and co-workers[16–18] have previously shown by polarographic measurements of O_2 uptake that the inhibition of endogenous respiration in Ehrlich hyperdiploid ascites tumor cells is partially relieved upon addition of glucose, indicating that reducing equivalents can enter the respiratory chain beyond the rotenone-sensitive site. The operation of both the α-glycerophosphate shuttle and a dicoumarol-sensitive pathway have been invoked to explain this glucose-dependent respiration[14]. We have tried to investigate whether these pathways were common to other strains of ascites tumor cells, by using the Lettre' mutant. In Fig. 1, glucose addition to rotenone-inhibited cells (A) is not able to stimulate the respiration unless vitamin K_3 (B) is present in the system. Thus, although reducing equivalents are formed after the addition of glucose (as independently established by NAD(P)H measurements), they cannot enter the respiratory chain, in the presence of rotenone, presumably due to the lack of a suitable shuttle system in this strain.

Redox changes of cytochromes b and c in rotenone-inhibited cells

Fig. 2 illustrates the response to the consecutive additions of rotenone, glucose and dicoumarol, of cytochrome *b* in cells utilizing only endogenous substrates in an experiment similar to that of Fig. 1A. 4.6 μM rotenone caused an oxidation of cytochrome *b*, measured as a decreased absorbance at 430–410 mμ. On addition of 2.3 mM

glucose there was a further oxidation and a new steady state was reached which lasted about 20 sec. Then followed a slow deflection in the opposite direction until 70 % of the cytochrome *b* was again reduced. These changes in cytochrome *b* occurred

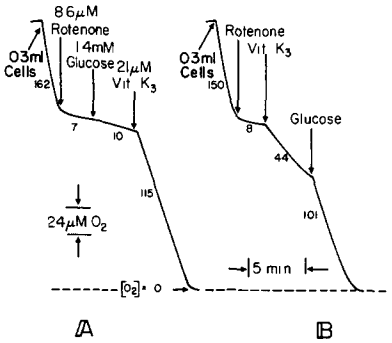
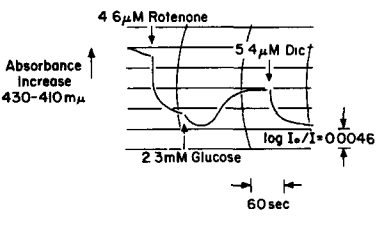


Fig 1 Polarographic recordings of O_2 uptake showing the effects of glucose and vitamin K_3 on the respiration inhibited by rotenone in ascites tumor cells The sequence of additions is different in the two traces, A and B When glucose is added first (A) no effect on the respiration is observed and only the subsequent addition of vitamin K_3 is able to restore at a large extent the respiration blocked by rotenone Vitamin K_3 alone activates the rotenone-inhibited respiration (B) by draining the intramitochondrial oxidation of endogenous reducing equivalents Glucose enhances the vitamin K_3 effect 0 3 ml of cells was added to 3 2 ml of saline–phosphate medium (pH 7 4) (for composition see MATERIALS AND METHODS) The final cell suspension concentration was 6 7 mg dry wt./ml The numbers along the traces indicate O_2 consumption in nmoles/min.

despite the absence of an alteration in respiratory rate (Fig. 1A). Subsequent addition of 5.4 μM dicoumarol reversed completely the effect induced by glucose. Oligomycin similarly reversed the reduction induced by glucose. In contrast to the cytochrome *b* response, cytochrome *c* only underwent oxidation on addition of glucose to rotenone-inhibited cells, without any subsequent reduction. This was shown by simultaneous measurements of cytochromes *b* (430–410 mμ) and *c* (550–540 mμ) and by



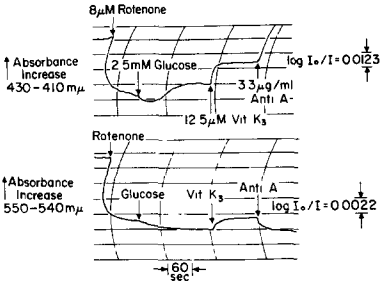


Fig 2 Spectrophotometric recording of the oxidation–reduction cycle of cytochrome *b* induced by glucose in ascites tumor cells inhibited by rotenone The cells were suspended in the saline–phosphate medium at the final concentration of 10 8 mg dry wt /ml Dic, dicoumarol

Fig 3 Simultaneous double-beam measurements of cytochromes *b* and *c* Addition of glucose to rotenone-inhibited cells causes an oxidation–reduction cycle of cytochrome *b* (*cf* Fig 2) while elicits only a slight oxidation of cytochrome *c*. Vitamin K_3 reduces both the cytochromes. Subsequent addition of antimycin A shows the cytochrome *b*–*c* crossover point The cell suspension concentration was 17 mg dry wt /ml.

low-temperature difference spectra of samples taken from the spectrophotometric cuvette during measurements of cytochrome *b* before ("reference") and after ("measure") the addition of glucose.

Fig. 3 shows the response of both the cytochromes *b* and *c* to the addition of glucose to rotenone-inhibited cells, as recorded simultaneously with the double double-beam spectrophotometer. The effects of vitamin K_3 and antimycin A are also presented in this figure. Rotenone, as expected, caused oxidation of the two cytochromes. The subsequent addition of 2.5 mM glucose provoked different effects on the cytochromes *b* and *c*, namely an oxidation–reduction cycle (similar to that shown in Fig. 2) of cytochrome *b* and a slight oxidation of cytochrome *c*. Addition of vitamin K_3, which is able to carry out the transfer of reducing equivalents from the cytosol to the respiratory chain[27], caused the reduction of both the cytochromes. Further addition of antimycin A clearly shows its specific action on one of the three crossover points of the mitochondrial respiratory chain, *i.e.*, that located between cytochromes *b* and *c*.

Low-temperature difference spectra of samples chilled before and after addition of glucose with the procedure indicated above, showed negative peaks for cytochrome *c* in the α and γ region (547 and 417 mμ respectively) and a positive peak for cytochrome *b* in the γ region with a maximum at 427 mμ. A positive shoulder is also present in the α region of cytochrome *b* at about 558 mμ.

Even if the spectral changes are rather small for giving clear informations on the nature of the pigment, the position of the γ band of cytochrome *b* and the apparent absence of the characteristic splitting of the α band into two peaks, which is typical of cytochrome b_5 at low temperature, may be indicative that the microsomal cytochrome b_5 is not concerned in the reduction induced by glucose in rotenone-inhibited cells

CHANCE AND MAITRA[28] have previously observed that in ascites tumor cells inhibited by amytal and sulfide the addition of glucose caused redox changes of cytochromes (oxidation of cytochrome oxidase and cytochrome *c*). The effect of glucose was interpreted as due to the increase of the phosphate potential ($ATP/ADP \cdot P_i$) in the cell which affected the inhibited respiratory chain by the reversal of electron transport. Under our conditions also (*i.e.*, even in absence of terminal inhibitors of electron transfer) the redox changes of cytochromes *b* and *c* during glycolysis could be caused by the same mechanism. An initial decrease of the phosphate potential due to glucose phosphorylation (causing oxidation of both cytochromes *b* and *c*) is, indeed, followed by an increase due to ATP formation in the cytosol (as indicated by subsequent reduction of cytochrome *b*). In order to establish whether this hypothesis was correct, the glucose effect on the redox changes of cytochrome *b* was studied in more detail. If 2-deoxyglucose was used instead of glucose, or if glucose was added to cells preincubated with iodoacetate or NaF (*i.e.*, under conditions where phosphorylation of the sugar is permitted, with initial decrease in ATP level, but no further or diminished ATP formation in the glycolysis is achieved), cytochrome *b* did not undergo the complete oxidation–reduction cycle, but only the oxidation was observed. The effect of iodoacetate and NaF, as well as that of other agents upon the oxidation–reduction cycle of cytochrome *b*, is presented in Table I. The initial oxidation of cytochrome *b* was inhibited by pretreatment with oligomycin and TTFB, whereas it was not affected by the other inhibitors. In the presence of sodium pyruvate a very fast reduction of cytochrome *b*, and no oxidation, was observed (see also below). The

TABLE I

EFFECT OF PRETREATMENT WITH DIFFERENT AGENTS UPON THE OXIDATION–REDUCTION CYCLE OF CYTOCHROME *b* INDUCED BY GLUCOSE IN ROTENONE-INHIBITED CELLS

5 μM (Expt. 1) or 8.3 μM rotenone and 2.5 mM (Expts. 1 and 2) or 250 μM glucose were added consecutively in all the cases to the spectrophotometric cuvette, containing 1.2 ml of cell suspension, and the absorbance changes recorded Substrates or inhibitors were added, when indicated, before (iodoacetate, NaF) or after rotenone. The final dry weights for 1 ml of cell suspension were the following. 16.2 (Expt 1), 14.5 (Expt 2), 14 4 (Expt. 3) and 13.2 mg (Expts. 4 and 5) The final concentration of the agents added were. 2.5 mM iodoacetate, 16 5 mM NaF, 0 83 mM sulfide, 8 3 μg/ml oligomycin, 7.2 μM TTFB, 5 μg/ml antimycin A and 4.2 mM sodium pyruvate. The cytochrome *b* concentration was calculated by using $\Delta\varepsilon$ (430–410 mμ) = 180 $mM^{-1} \cdot cm^{-1}$

Expt. No	*Substrate or inhibitor*	*Cytochrome b (nmoles/g dry wt.)*	
		Oxidation	*Reduction*
1	None	2 4	8.6
	Iodoacetate	3.6	0 0
2	None	2 2	5 1
	NaF	4 4	0 0
	S^{2-}	2.2	14 7
	NaF + S^{2-}	2.9	0 0
3	None	2.9	6 8
	Oligomycin	1.4	1.4
	TTFB	0.0	0.0
4	None	1.9	4 7
	Antimycin A	1 4	0 0
5	None	2.3	4.7
	Pyruvate	—*	2 4

* Pyruvate pretreatment caused a change in the glucose response the oxidation was abolished and substituted by a very fast reduction, complete in the mixing time

reduction phase of the cytochrome *b* cycle was suppressed not only by glycolytic inhibitors, such as iodoacetate or fluoride, but also by TTFB, antimycin A and inhibited 80 % by oligomycin and 50 % by pyruvate.

From these findings it seems possible to conclude that the oxidation–reduction cycle of cytochrome *b* proceeds parallel to the transition from the forward to the reversed electron transfer, caused by the change in the phosphate potential which was initially lowered and then increased upon glucose addition.

Redox changes of cytochromes b and c in presence of vitamin K_3

In ascites tumor cells vitamin K_3 is able to establish an electron shunt between extramitochondrial pyridine nucleotides and the respiratory chain *via* the flavoprotein DT-diaphorase (EC 1.6.99.2) ("vitamin K_3 shunt")[29].

Figs. 4 and 5 present kinetic experiments of cytochromes *b* and *c* after the activation of the DT-diaphorase pathway by vitamin K_3. The traces of Figs. 4A and 5A show that upon addition of 21 μM vitamin K_3 there was an abrupt reduction of cytochromes *b* and *c* which was followed by a slower restoration of the original oxido-reduction state. In parallel measurements of pyridine nucleotide fluorescence (not shown), it was observed that vitamin K_3 also caused an oxidation of reduced pyridine

nucleotides. The addition of glucose after vitamin K_3 caused a biphasic reduction (a fast and a slow phase) of cytochromes *b* and *c* (less evident) which was increased in extent, and modified in its kinetics, when oligomycin had previously been added. TTFB relieved the effect induced by oligomycin and inhibited the glucose effect itself

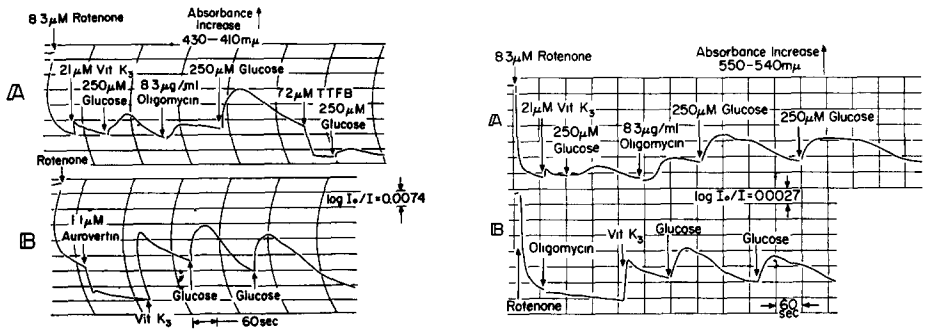


Fig. 4 Different response of cytochrome *b* in the presence or absence of inhibitors of energy transfer (oligomycin, aurovertin, TTFB) to the addition of vitamin K_3 or vitamin K_3 *plus* glucose in ascites cells treated with rotenone. Cell suspension concentration was 17.1 mg dry wt./ml The abrupt downward deflection upon addition of aurovertin was due to the absorbance of this substance in the region of spectroscopic measurement.

Fig. 5 Effect of oligomycin on the response of cytochrome *c* in an experiment comparable to that of Fig. 4. Cell suspension concentration was 17.3 mg dry wt /ml.

TABLE II

EFFECT OF PRETREATMENT WITH DIFFERENT AGENTS UPON THE VITAMIN K_3-MEDIATED REDUCTION OF CYTOCHROME *b* INDUCED BY GLUCOSE IN ROTENONE-INHIBITED CELLS

8.3 μM rotenone, 21 μM vitamin K_3 and 250 μM glucose were present in all the experiments. They were added consecutively to the spectrophotometric cuvette, containing 1.2 ml of cell suspension. Substrates and inhibitors were added at the same concentrations indicated in Table I, before rotenone (iodoacetate, NaF) or after rotenone and vitamin K_3 (oligomycin, TTFB, pyruvate). The final dry weights for 1 ml of cell suspension were the following: 14.1 (Expt. 1), 14.6 (Expt. 2) and 14.4 mg (Expt. 3)

Expt.	*Substrate or inhibitor*	*Cytochrome b reduction (nmoles/g dry wt)*	
		Fast	*Slow*
1	None	1.8	4.1
	Iodoacetate	0.0	0.0
2	None	3.7	2.7
	NaF	3.7	0.0*
3	None	2.2	1.9
	Oligomycin	9.3	
	TTFB	1.3	0.0
	Oligomycin + TTFB	3.2	0.8
	Pyruvate	5.4	2.2
	TTFB + pyruvate	4.3	0.0
	Oligomycin + TTFB + pyruvate	5.4	1.1

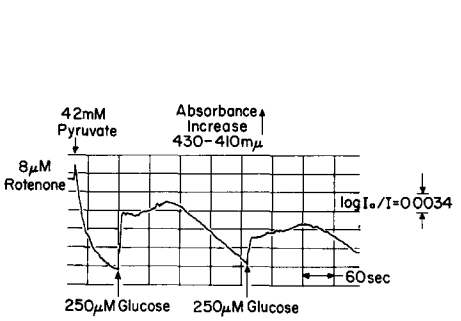
* With high glucose concentrations (2.5 mM) the inhibition is 70%.

(Fig. 4A). The reduction of cytochromes *b* and *c* induced by vitamin K_3 alone was also increased (from 4 to 5 times) by oligomycin or aurovertin (Figs. 4B and 5B). Table II reports the effects of pretreatment with different agents upon the vitamin K_3-mediated reduction of cytochrome *b* in the presence of glucose. The fast reduction of cytochrome *b* induced by glucose in the presence of vitamin K_3 was completely inhibited by iodoacetate and 40 % by TTFB; it was unaffected by NaF and markedly increased by pyruvate. The slow reduction was inhibited by iodoacetate, NaF and TTFB while it was unchanged by the pyruvate addition. These data suggest that the feeding of reducing equivalents from the extramitochondrial compartment to the respiratory chain follows closely the kinetics of glucose uptake. The slowing down of cytochrome *b* reduction a few seconds after the glucose addition can, indeed, be explained by the reported inhibition of glycolysis by vitamin K_3 in the presence of amytal or rotenone[8,29]. The increase in the rate of reduction of cytochromes *b* and *c* in the presence of oligomycin is probably caused by the reduced electron flow. However, an inhibitory effect of oligomycin on the O_2 uptake induced by vitamin K_3, which could support this hypothesis, was not observed. Thus the mechanism of the oligomycin effect is still open to other explanations

The effect of pyruvate addition on the redox level of cytochromes in rotenone-inhibited cells in the presence and absence of glucose

Having established that no reducing equivalents are transported between cytoplasm and mitochondria in the presence of rotenone in this strain, unless vitamin K_3 is present, we have tried to investigate the pathways of intracytoplasmic hydrogen transport

Fig. 6 shows the effect of pyruvate on the oxidation–reduction cycle of cytochrome *b* induced by glucose in rotenone-inhibited cells. As described in Fig. 2, pretreatment with 8 μM rotenone (not shown) caused a large oxidation of cytochrome *b**; pyruvate, added after rotenone, oxidized this pigment further (6.2 nmoles/g dry



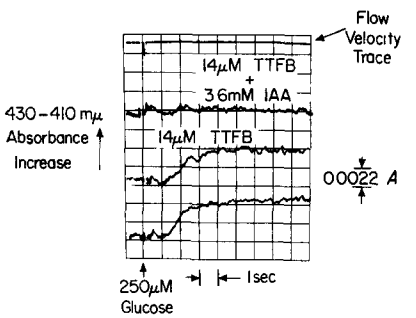


Fig 6 Effect of pyruvate upon the response of cytochrome *b* to glucose addition in rotenone-inhibited cells Cell suspension concentration was 14 6 mg dry wt /ml

Fig 7 Rapid-flow apparatus traces showing the effect of additions of 250 μM glucose to rotenone- and pyruvate-treated cells The fast reduction by glucose ($t_{1/2}$ 680 msec) at 430–410 mμ is inhibited by iodoacetate (IAA, top trace) but not by the uncoupler TTFB (middle trace) The final cell concentration was 16 7 mg dry wt /ml The suspension was bubbled with O_2 before the addition of rotenone (10 7 μM) and pyruvate (16 mM)

* The concentration of cytochrome *b* present in the respiratory chain, calculated from difference spectra between anaerobic cells and rotenone-treated aerobic cells, was found, to be 21 nmoles/g dry wt. Rotenone gave an oxidation of cytochrome *b* of 17 9 ± 0.3 (5) nmoles/g dry wt. (mean ± S E , number of observations in parentheses).

wt.) indicating that it was not completely oxidized when the inhibitor alone was present. Cytochrome *c* also showed an oxidation upon addition of pyruvate to rotenone-treated cells, but in this case the response was very slight. As observed by measurements of pyridine nucleotides fluorescence (not shown) pyruvate added after rotenone induced an oxidation of pyridine nucleotides owing to the reaction with NADH at the lactate dehydrogenase (EC 1.1.1.27) level Therefore it seems likely that the steady-state reduction of the cytochrome *b* component, measured at 430–410 mμ, is maintained not only by NAD-linked endogenous substrates, whose oxidation is inhibited by rotenone, but also by the extramitochondrial NADH through a pathway which is insensitive to rotenone.

The addition of glucose after pyruvate induced a response of cytochrome *b* which was different from that produced when pyruvate was absent. Fig. 6 shows this difference. Upon addition of glucose a fast reduction was observed, followed after a lag of about 30 sec, by a much slower reduction. The effect of glucose was transient due to the low concentration added (250 μM) After about 4 min all glucose was used up and the oxidized steady state obtained upon addition of pyruvate was again reached. A second addition of glucose produced the same biphasic response but the initial fast reduction was decreased by about 50 % Cytochrome *c* measured under the same conditions, underwent changes which, although qualitatively identical, were again much smaller than those of cytochrome *b*.

TABLE III

DIFFERENT RESPONSES OF A CYTOCHROME *b*-TYPE PIGMENT AT TWO DIFFERENT WAVELENGTH PAIRS

The conditions were similar to that of the experiment of Fig 6 The cells (13 5 mg dry wt /ml) were preincubated with 8 μM rotenone *Minus* indicates cytochrome oxidation, *plus* indicates cytochrome reduction.

Additions	*Δ Absorbance*	
	430–410 mμ	*425–410 mμ*
Pyruvate (4 2 mM)	−0 0188	−0 0244
Glucose (250 μM)	+0 0085 (fast) +0 0056 (slow)	+0 0141 (fast) +0 0028 (slow)

In Table III an experiment similar to that of Fig. 6 shows how a change in the wavelength pair from 430–410 to 425–410 mμ caused an increase in the extent of oxidation by pyruvate and of the fast reduction by glucose, while the slow reduction decreased.

In Fig. 7 an experiment, performed with the regenerative flow apparatus, shows the kinetics of the cytochrome reduction at 430–410 mμ upon addition of glucose to rotenone- and pyruvate-treated cells. The bottom trace shows how a pulse of 250 μM glucose, after a lag of 1 sec, caused an increase in absorbance which was almost complete in about 3 sec. The half-time of this reaction was 680 msec and the cytochrome reduced corresponded to 2.5 nmoles/g dry wt. TTFB (middle trace) caused a change in the half-time (950 msec) without changing the extent of reduction. On the other hand, pretreatment with iodoacetate (top trace) completely abolished the glucose effect.

TABLE IV

EFFECT OF PRETREATMENT WITH INHIBITORS AND UNCOUPLERS UPON THE REDUCTION OF A CYTOCHROME *b*-TYPE PIGMENT INDUCED BY GLUCOSE IN THE PRESENCE OF ROTENONE AND PYRUVATE

8.3 μM rotenone, 4 2 mM sodium pyruvate and 250 μM glucose were present in all the experiments. They were added consecutively to the spectrophotometric cuvette, containing 1.2 ml of cell suspension Inhibitors and uncouplers were added, at the same concentration indicated in Table I, before rotenone (iodoacetate and NaF) or after rotenone and pyruvate (oligomycin, TTFB and antimycin A) The final dry wts. were 14 1, 14.4 and 16 3 mg/ml for Expts 1, 2 and 3, respectively.

Expt	*Inhibitor or uncoupler*	*Reduction at 430–410 mμ (nmoles/g dry wt.)*	
		Fast	*Slow*
1	None	2 4	3 0
	Iodoacetate	0 0	0 0
	NaF	3 0	1 5
2	None	—*	—*
	Oligomycin	3 2	0 0
	TTFB	3 5	0 0
3	None	2 6	2 2
	Antimycin A	3.8	0 0

* Not tested

For completeness, Table IV also shows the effect of some inhibitors and uncouplers upon the reduction induced by glucose, in the presence of rotenone and pyruvate, of pigment(s) measured at 430–410 mμ. The fast reduction was inhibited by iodoacetate but not by NaF, oligomycin, TTFB and antimycin A. The slow reduction, on the other hand, was inhibited by all the inhibitors tested.

DISCUSSION

Intracellular hydrogen transport and aerobic glycolysis

The problem of the reoxidation of glycolytic reducing equivalents in tumor cells seems to be of particular interest because of the usual high rate of aerobic glycolysis in these cells. WEINHOUSE[7] first pointed out the importance of this problem. The failure to find certain enzyme activities supposedly involved in shuttle mechanisms for the intramitochondrial translocation of glycolytic reducing equivalents, suggested that the high rate of aerobic lactate production in tumor cells is due to the ineffectiveness of the mitochondria in the oxidation of the reducing equivalents synthesized in the cell sap[2,8]. However, this conclusion may be challenged at the present time on the basis of the following considerations: (a) the lack of key enzymes for the proposed shuttle mechanisms between the cytosol and the mitochondrial respiratory chain (beyond the first phosphorylation site), such as α-glycerophosphate dehydrogenase, is not a common feature of all the kinds of tumor tissues with a high rate of aerobic glycolysis[13–15]; (b) in any case, there is evidence from experiments with vitamin K_3 (ref. 29), that intramitochondrial oxidation of glycolytic reducing equivalents, beyond the first phosphorylation site, may not affect the aerobic production of lactate to any great extent; (c) pathways of intramitochondrial oxidation through the "inter-

nal" NADH have been proposed in ascites tumor cells[3,30] and, finally, (d) the reoxidation of reducing equivalents may not be limited to the mitochondrial electron transport chain, but may also include the microsomal chain.

Intramitochondrial oxidation of reducing equivalents in the presence of rotenone

We have investigated the intracellular hydrogen transfer in intact Ehrlich ascites tumor cells, in the attempt to corroborate some of the considerations stated above. The general conclusions on some of the metabolic relationships between the extramitochondrial and the mitochondrial space as well as those between different compartments within the cytosol are represented schematically in Fig. 8. The cells were inhibited by rotenone in order to prevent the oxidation of endogenous NAD-linked substrates. Addition of glucose to these cells did not show intramitochondrial transfer of reducing equivalents to O_2, but only a reversal of electron transfer that was presumably induced by the increase in the cytoplasmic phosphate potential. The partial reactivation by glucose of the rotenone inhibition of the endogenous respiration, found by other authors[16–18] in Ehrlich ascites cells, is probably due to the presence of shuttle mechanisms which are lacking in the strain used for the present study.

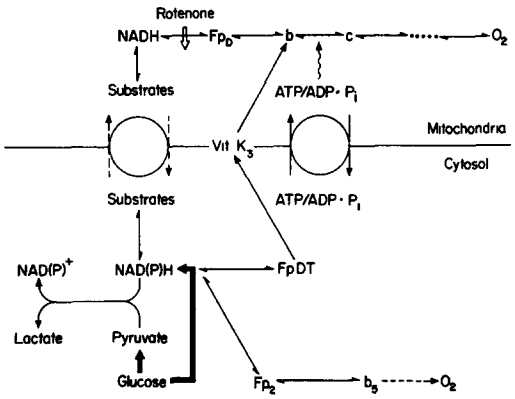


Fig 8 Diagrammatic representation of pathways of hydrogen transport and cytosolic–mitochondrial interactions in Ehrlich ascites tumor cells. See text for explanations. ⟹, multistep pathways, ⟶, simple pathways; - - -→, possible pathways, ⇝, interactions, ⟾, inhibitor site. Fp_D, NADH dehydrogenase (EC 1. 6.99 3); FpDT,DT-diaphorase; Fp_2, NADH–cytochrome b_5 reductase (EC 1.6 2.2).

These findings stress the point that a generalization on the absence of pathways for the transfer of cytoplasmic hydrogen to the electron transfer chain beyond the rotenone-inhibited site, is not possible for all kinds of Ehrlich ascites carcinoma cells. That, in our conditions, reducing equivalents are formed after addition of glucose to rotenone-inhibited cells is demonstrated both by the increase in the redox state of nicotinamide nucleotides upon glucose addition and by the experiments with vitamin K_3. Vitamin K_3 is able to carry out the transfer of reducing equivalents from the cytosol to the respiratory chain, as shown by the stimulation of the respiration and by the steady-state changes of respiratory carriers. These data confirm the earlier observation of ERNSTER and co-workers[8,29] on the effect of vitamin K_3 in Ehrlich ascites cells.

No evidence is presented in this paper for or against the suggestion of other authors[3,30] about the existence in these cells of pathways for intramitochondrial oxidation of cytoplasmic reducing equivalents *via* NAD-dependent substrate couples, such as acetoacetate–β-hydroxybutyrate and oxalacetate–malate. However, the experiments now to be discussed paid some attention to the further possibility that reoxidation of cytoplasmic reducing equivalents may also take place in organelles other than the mitochondria.

Extramitochondrial hydrogen transport

The addition of pyruvate to rotenone-inhibited cells blocked the transfer of endogenous reducing equivalents to a cytochrome *b*-type pigment, but this was partly restored by a subsequent addition of glucose (see Fig. 6). The contribution of extramitochondrial pyridine nucleotides to the maintenance of the steady-state reduction of this cytochrome was about one third of that due to NAD-linked mitochondrial electron transport. The following experimental observations suggest that the absorption changes observed in the type of experiment shown in Fig. 6 may be mainly due to cytochrome b_5 and only for a small part to cytochrome *b*. (i) The response of the cytochrome *b* component was greater by 30 and 65 % for the oxidation by pyruvate and the subsequent fast reduction by glucose respectively, when the wavelength pair 425–410 was used instead of 430–410 mμ (see Table III). (ii) The extent of the fast reduction of the cytochrome *b*-type pigment by glucose was not diminished by treatment with oligomycin, with TTFB, or by the prior reduction of the respiratory chain cytochrome *b* induced by antimycin A (see Table IV). (iii) Cytochrome *c* showed only a slight response upon the addition of pyruvate and glucose to rotenone-inhibited cells.

The kinetic data obtained from the rapid-flow technique (*cf.* Fig. 7) also support the idea of a role of cytochrome b_5, since the half-times obtained are in the range of those reported by COOPER *et al.*[31] for reduction of cytochrome b_5 by NADH in isolated microsomes. On the other hand the slow reduction by glucose was abolished by oligomycin, TTFB and antimycin A and was decreased by about 50 % when measured at 425–410 mμ, indicating the preferential involvement of cytochrome *b* in this reaction. The increase in the phosphate potential subsequent to the glucose addition can explain how this reaction is caused by a reversal of electron transfer. This slow reduction was half that obtained in the absence of pyruvate, probably due to the inhibition of glycolysis by pyruvate[32].

At this point the following conclusion can be drawn. When the oxidation of endogenous substrates is prevented by rotenone, the addition of excess pyruvate to the cells allows a titration of cytoplasmic NADH to be made *in vivo*. The reduced pyridine nucleotide is presumably distributed in different compartments within the cytoplasm, partly in the free form and partly bound to dehydrogenases[33]. Among these dehydrogenases, those involved in the mixed function oxidase reactions of the microsomal fraction seem to play a part in causing the reoxidation of NADH. This is demonstrated by the effect of glucose when added after pyruvate. About 60 % of the cytochrome oxidized by pyruvate, as measured at 430–410 mμ, is, indeed, reduced by glucose. This cytochrome can be almost entirely identified with cytochrome b_5 localized in the endoplasmic reticulum, and to a small extent with a mitochondrial cytochrome of the *b* type in functional relation to cytochrome *c*. That the amount of

cytochrome b_5 *plus* cytochrome *b* reduced by glucose is less than that originally oxidized, may be explained by the fact that part of the NADH generated during the glycolysis is reoxidized by the lactate dehydrogenase reaction.

Thus these measurements can give information on the contribution of two different pathways to the regulation of the redox level of NADH in the cytosol when glycolysis occurs and when the respiratory chain is blocked at the first phosphorylation site. However, the system used here represents an over-simplification of the mechanisms operating under physiological conditions, since, in the presence of rotenone, no account is taken of the possible contribution of the NADH→flavoprotein portion of the intramitochondrial electron transfer chain to the reoxidation of cytosolic NAD(P)H. If, indeed, as mentioned above, the contribution of flavin-linked shuttle mechanisms in this strain of ascites tumor cells is absent or very low, the possibility, however, that translocation of glycolytic reducing equivalents into the mitochondria occurs through the "internal" NADH cannot be ruled out.

Finally we have, thus, tried to see whether the coexistence of different pathways for NAD(P)H oxidation could occur in these cells by using the combination of pyruvate and vitamin K_3. It was seen that microsomal reoxidation of NADH was not prevented by the active operation of an artificial intramitochondrial transfer of reducing equivalents. This was shown by the increased extent of reduction induced by glucose at 430–410 mμ when both vitamin K_3 and pyruvate were present, in comparison with the system containing only vitamin K_3 (*cf.* Table II).

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