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Interrelation between mitochondrial respiration, substrate supply and redox ratio in perifused permeabilized rat hepatocytes

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Abstract

A one-step perifusion technique is described for studying the regulation of energy metabolism in intact hepatocytes and in mitochondria of the same cells after their permeabilization by digitonin. Cell count and activities of glutamate dehydrogenase, the latter being used as an indicator of mitochondrial integrity, were found to be nearly unchanged after permeabilization and perifusion for at least 40 min at 37°C. The residual activity of lactate dehydrogenase after permeabilization indicated that permeabilized cells were almost depleted of soluble cytosolic components. The composition of the perifusion medium was chosen so that various metabolic states could be adjusted of both intact and permeabilized hepatocytes without the need to change the perifusion medium. Oxidative phosphorylation of mitochondria within permeabilized hepatocytes remained intact throughout the perifusion as indicated by the response of respiration to the addition of ADP, carboxyatractyloside and uncoupler. The application of the perifusion technique allows us to sample indicator metabolites in the effluent medium like acetoacetate (AcAc) and 3-hydroxybutyrate (HB) for calculating the mitochondrial redox ratios and rates of ketogenesis. In the presence of octanoate and ADP, an improvement of substrate supply by glutamate and malate led to increases in the intramitochondrial HB/AcAc ratio and the respiration rate. Glutamate/malate concentrations of 1 mM resulted in maximal respiration rates, whereas concentrations of 5 mM further enhanced the HB/AcAc ratio. Mitochondria responded to increasing ATP/ADP ratios in the perifusion medium by decreased respiration rates at higher HB/AcAc ratios. By comparing respiration rates and redox ratios of mitochondria in permeabilized cells with those before permeabilization (gluconeogenic conditions of hepatocytes), it is concluded that in the intact cell oxidative phosphorylation is limited with respect to substrate supply as well as by the ATP demand.

Keywords: Bioenergetics; Respiration rate; Hepatocyte; Mitochondrion; Perifusion; (Permeabilized cell)

1. Introduction

Oxidative phosphorylation is by far the most important energy-conserving pathway, supplying the cell with ATP to enable a wide range of energy consuming processes. Therefore, there is a special interest in the mechanisms controlling this process. Today, there is a consensus that oxidative phosphorylation, for example in the liver, is mainly controlled by ATP consuming processes such as gluconeo- and ureogenesis (for a recent review see Ref. [1]). It has been shown that the rates of gluconeogenesis and ureogenesis depend on both the supply of precursors such as lactate, pyruvate or alanine as well as the presence of additional oxidizable substrates to enable ATP production [2]. The latter process can be activated via hormonal signalling followed by enhanced Ca^{2+} release and therefore leading to an activation of some matrix dehydrogenases, i.e., pyruvate dehydrogenase, (NAD^+)-isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase [3–5]. In a rather simple system such as isolated mitochondria, oxidative phosphorylation is not simply dependent on extramitochondrial ADP supply but is primarily controlled by the extramitochondrial ATP/ADP ratio which is also modified by the substrate hydrogen supply [6,7]. Nevertheless, investigation of the interrelation between oxidative phosphorylation and substrate supply of isolated mitochondria under incubation conditions has some limits, because decreasing the substrate concentration could lead to non-stationary conditions.

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; GLDH, glutamate dehydrogenase (EC 1.4.1.3); LDH, lactate dehydrogenase (EC 1.1.1.27).

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We have presented a method to permeabilize hepatocytes by digitonin using a perifusion technique [8]. The advantage of this method is that after addition of digitonin to the perifusion chamber, the excess amount of digitonin and the soluble cytosolic components could be easily washed out by continuous perifusion while no centrifugation steps are needed. After transferring such permeabilized cells from the perifusion chamber to an oxygraphic incubation chamber, mitochondria could be demonstrated to show characteristics within these permeabilized cells as they would have in the isolated state.

The present study demonstrates that perifusion of hepatocytes can be continued after permeabilization by digitonin resulting in functionally intact mitochondria. Using the advantage of this experimental approach to adjust stationary states, respiration and redox state should be measured to characterize the mitochondrial activity. To consider the full range of this activity the substrate supply as well as the energetic load were varied in the perifusion medium. The resulting relation between respiration and the redox state should be used to characterize the energetic situation of mitochondria within the intact cells before permeabilization.

2. Materials and methods

2.1. Biochemicals

ADP and ATP were obtained from Reanal (Budapest, Hungary); carboxyatractyloside, collagenase, and FCCP from Boehringer (Mannheim, Germany); 3-hydroxybutyrate dehydrogenase from Universität Leipzig (Germany), and digitonin from YSAT (Wernigerode, Germany). All other chemicals were of analytical grades.

Solutions used were: buffer A for preparation of hepatocytes consisting of 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, and 1.25 mM $CaCl_2$, adjusted to pH 7.4 [9], and gassed with carbogen (95%O_2, 5%CO_2); and buffer B for the combined perifusion of intact and permeabilized hepatocytes consisting of 50 mM mannitol, 50 mM Tris-HCl, 40 mM KCl, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 0.5 mM EDTA, and 20 mM $NaHCO_3$, adjusted to pH 7.4.

2.2. Isolation of hepatocytes

Male Wistar rats (180–250 g) starved for 24 h were used throughout. Hepatocytes were prepared as described by Berry and Friend [10] with the modifications reported by Seglen [11]. Isolated cells were stored in buffer A at 0–4°C. The dry weight of the stock solution was about 30 mg per ml, the minimum viability of cells 85% based on Trypan blue exclusion [12].

2.3. Perifusion and permeabilization of hepatocytes

Isolated hepatocytes were perifused by the technique of Van Der Meer and Tager [13] with several modifications (Fig. 1). The total volume of the perifusion chamber was 6.5 ml. Type SM-11301 membrane filters of 8 μm pore size were purchased from Sartorius (Göttingen, Germany). The buffer used for perifusion was gassed at 37°C with carbogen using an oxygenator as proposed by S. Soboll (personal communication). Cells were perifused at a flow rate of 8 ml per minute.

Hepatocytes were permeabilized with digitonin given directly into the perifusion chamber.

With this approach, 5 μg digitonin per mg dry weight were sufficient to permeabilize cells nearly completely at 37°C. The dry weight of hepatocytes was calculated via packed cell volume which was obtained after centrifugation of 100 μl of stock solution at 1800 × g for 15 min. The ratio between packed cell volume (μl/ml) and dry

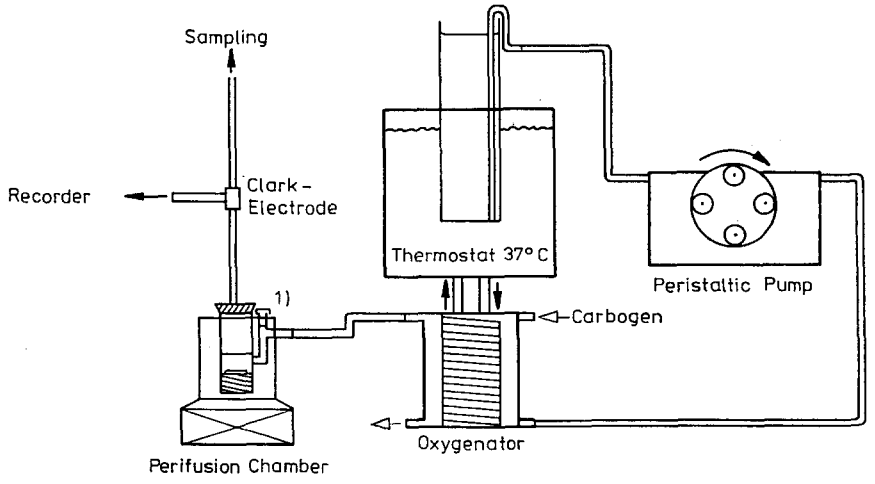

Fig. 1. Experimental set-up for the perifusion of intact and permeabilized rat hepatocytes. (1) Filling port for cells, digitonin, and effectors such as carboxyatractyloside or FCCP.

Table 1
Cell count, Trypan blue exclusion (TBE), LDH and GLDH activities of intact and permeabilized hepatocytes

	Experiment	Cell count	Cells (%)	TBE (%)	LDH	LDH (%)	GLDH	GLDH (%)
Intact cells	1.0	15.6	100.0	87	72.4	100.0	26.2	100.0
Permeabilized cells	1.1	13.4	85.9	10	6.4	8.8	26.2	100.0
	1.2	15.0	96.2	10	8.2	11.3	25.9	98.8
	1.3	13.0	83.3	8	5.5	7.6	25.9	98.8
	1.4	14.1	90.4	12	7.3	10.1	23.8	90.8
Intact cells	2.0	13.2	100.0	86	41.4	100.0	23.1	100.0
Permeabilized cells	2.1	13.2	100.0	10	6.4	15.5	21.1	91.3
	2.2	12.5	94.7	10	4.5	10.9	22.4	97.0
	2.3	9.6	72.3	7	3.7	8.9	23.1	100.0
	2.4	12.2	92.4	10	5.5	13.3	22.4	97.0

Note. Cells were perifused with medium B (for composition of the medium see Methods section) which was complemented by 1 mM lactate, 0.1 mM pyruvate, 0.1 mM octanoate, 1 mM glutamate and 1 mM malate at 37°C. After their permeabilization by adding digitonin (5 μg/mg dry weight), ADP (0.38 mM) was added and perifusion was continued for 40 min. Then permeabilized cells were taken out of the chamber and analysed (see Methods section). Cell count is given as 10^6 cells/ml, enzyme activities are given in U/ml cell suspension within the perifusion chamber.

weight (mg/ml) was found to be 0.28 ± 0.02 μl per mg dry weight [8].

2.4. Assays

Oxygen consumption was measured with a Clark-type electrode at 37°C.

GLDH and LDH as well as 3-hydroxybutyrate, acetoacetate and adenine nucleotides were determined by standard spectrophotometric methods [14]. Enzyme activities were measured after treatment of intact or permeabilized cells with 0.5% Triton X-100 dissolved in the assay buffers. After centrifugation at $1800 \times g$ (1 min), the supernatants were used for the assay.

For counting cells in a hemocytometer, 100 μl of the hepatocyte stock solution were diluted with 400 μl of medium A or B containing 0.125% Trypan blue, permitting a simultaneous estimation of cell viability.

2.5. Statistics

Generally, statistical analyses of all data (including data of the assays) were carried out using the *U*-test (Mann-Whitney-Wilcoxon) or (if indicated) Wilcoxon's matched-pairs signed rank tests.

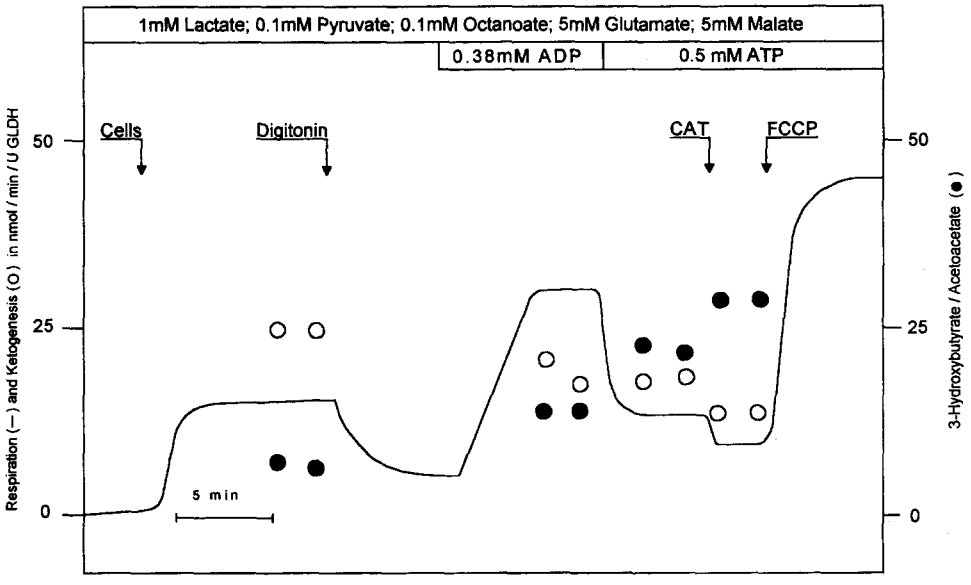

Fig. 2. Oxygraphic traces, rates of ketogenesis and 3-hydroxybutyrate/acetoacetate ratios of intact and permeabilized rat hepatocytes. Cells (30 mg dry weight) were perifused with medium B which was completed by lactate, pyruvate, octanoate, malate, glutamate, ATP and ADP as indicated. Digitonin was added in a concentration of 5 μg per mg dry weight. Carboxyatractyloside (CAT, 0.39 μmol) and FCCP (0.01 μmol) were injected directly into the perifusion chamber. The metabolic rates were expressed per unit (U) of GLDH (see Methods).

3. Results

Perifusion of permeabilized hepatocytes should meet both establishing real steady states of supplying mitochondria with metabolizable substrates and simple sampling of metabolites at the efflux site of the perifusion chamber. However, this requires morphological and functional stability of mitochondria within permeabilized hepatocytes for a sufficient time. Earlier findings [8] indicate that the cell count and the activity of glutamate dehydrogenase (a sensitive marker of the integrity of mitochondria, [15]) were nearly unchanged after permeabilization and storage in an ice bath. To test the stability of permeabilized cells under perifusion conditions, the flow was continued for 40 min after addition of digitonin. Normally, the basal media used to study isolated cells such as hepatocytes differ from those used to study isolated organelles such as mitochondria. For isolated cells, the Krebs/Ringer bicarbonate medium is well established [9], whereas for the latter a lot of media exist which are adapted to various intracellular conditions [6,16,17]. In order to avoid changing the medium during the permeabilization of hepatocytes, one medium was tested that should meet both extra- and intramitochondrial requirements. For this purpose the medium described by Küster et al. [18] to study isolated mitochondria was slightly modified. The main difference was the exchange of parts of potassium chloride and sucrose for sodium bicarbonate and mannitol, respectively (see Methods). Nedergard [19] found 20 mM sodium bicarbonate to be sufficient for normal hepatocyte function. In Table 1, the results obtained of two hepatocyte preparations are listed, each of which was used in four permeabilization experiments. Cell count and glutamate dehydrogenase activities are seen to be nearly unchanged at the end of the perifusion period. Permeabilized cells showed a Trypan blue exclusion and a recovery of lactate dehydrogenase activity of about 10% which indicates that permeabilized cells were nearly completely depleted of soluble cytosolic components.

To prove the functional integrity of mitochondria in permeabilized perifused cells, the capacity of oxidative phosphorylation was tested. In Fig. 2, a typical oxygraphic trace is shown for respiration before and after permeabilization of hepatocytes with the substrates lactate, pyruvate and octanoate for intact cells and, in addition, glutamate and malate for permeabilized cells. After the intact cells had reached a steady state of respiration, digitonin (5 μg/mg dry weight) was added to initiate permeabilization. Immediately after permeabilization, respiration decreased to a minimum rate. Glutamate dehydrogenase activity was used as a reference to compare the metabolic rates of both intact and permeabilized cells.

The addition of ADP stimulated respiration to a rate which was considerably higher (nearly doubled) than that obtained in intact cells. This increase of respiration indicated that the fall in respiration immediately after permeabilization was due to a loss of endogenous adenine nucleotides. An exchange of ADP for ATP resulted in a decrease of respiration indicating that respiration responds to changes in the extramitochondrial ATP/ADP ratio. This decrease was pronounced by addition of carboxyatractyloside to block the adenine nucleotide translocase. Because of its high affinity to adenine nucleotide translocase, carboxyatractyloside was added as a single injection directly into the perifusion chamber. After addition of the uncoupler FCCP, respiration was stimulated to a value which was higher than that obtained in the presence of ADP (+35%). The respiratory control ratio (RCR) calculated as ratio of ADP-stimulated to carboxyatractyloside-inhibited respiration rates was about 4.

Table 2
Respiration rate, respiratory control ratio (RCR), intramitochondrial redox ratio (HB/AcAc) and ketogenesis of permeabilized perifused hepatocytes with varied glutamate/malate concentrations

	Respiration rate (nmol O_2/min per U GLDH)	RCR	HB/AcAc	Ketogenesis (nmol/min per U GLDH)
Before permeabilization				
5 mM Glu, 5 mM Mal	20.0 ± 4.4		1.7 ± 0.3	12.1 ± 3.8
After permeabilization				
+0.38 mM ADP, $n = 6$	28.0 ± 6.2		6.4 ± 0.9	17.2 ± 4.2
+0.06 mM CAT, $n = 6$	8.2 ± 2.9	3.9 ± 1.8	13.3 ± 3.2	9.2 ± 3.7
Before permeabilization				
1 mM Glu, 1 mM Mal	20.8 ± 7.4		1.6 ± 0.5	11.6 ± 2.7
After permeabilization				
+0.38 mM ADP, $n = 8$	31.0 ± 5.6		1.5 ± 0.6 *	7.6 ± 2.6 *
+0.06 mM CAT, $n = 8$	8.8 ± 2.0	3.8 ± 1.3	6.2 ± 0.6 *	6.7 ± 0.9

Cells were perifused and permeabilized as in Fig. 2 with the exception that glutamate and malate were added to a final concentration of either 5 or 1 mM. RCR was calculated as the ratio between ADP-stimulated and carboxyatractyloside (CAT)-inhibited respiration rates. HB, 3-hydroxybutyrate; AcAc, acetoacetate. Data are given as means ± S.D.

* $P < 0.05$ (relative to similar perifusion conditions but with higher glutamate/malate concentrations, U-test).

Table 3
Respiration rate, respiratory control ratio (RCR), intramitochondrial redox ratio (HB/AcAc) and ketogenesis of permeabilized perifused hepatocytes with different sources of substrate hydrogen supply

	Respiration rate (nmol O_2/min per U GLDH)	RCR	HB/AcAc	Ketogenesis (nmol/min per U GLDH)
Before permeabilization				
1 mM Lac, 0.1 mM Pyr				
0.1 mM octanoate	20.6 ± 5.0		1.6 ± 0.4	12.7 ± 2.3
After permeabilization				
+0.38 mM ADP	12.6 ± 4.2 *		0.6 ± 0.3 *	8.4 ± 1.1 *
+1 mM Mal	30.9 ± 5.0 *		1.1 ± 0.6 *	6.0 ± 1.2 *
+1 mM Glu	35.4 ± 1.8 *		1.6 ± 0.8 *	8.4 ± 1.9 *
+0.06 mM CAT	10.8 ± 4.8 *	3.9 ± 1.6	5.2 ± 1.0 *	6.7 ± 1.5 *

Cells were perifused and permeabilized as in Fig. 2 with the exception that malate and glutamate were added successively after permeabilization to a final concentration of 1 mM each. RCR was calculated as the ratio between ADP-stimulated and carboxyatractyloside (CAT)-inhibited respiration rates. HB, 3-hydroxybutyrate; AcAc, acetoacetate. Data are given as means ± S.D., $n = 4$.
* $P < 0.05$ (relative to the preceding perifusion conditions, Wilcoxon test).

In the presence of fatty acids like octanoate as substrate, an additional estimation of the indicator metabolites 3-hydroxybutyrate and acetoacetate in the efflux of the perifusion chamber allowed a simple calculation of both the intramitochondrial redox ratio [20] and the rate of ketogenesis. This made it possible to study the interaction between varied hydrogen supply and respiration rate. In Table 2, a summary is given of several experiments performed in the same way as seen in Fig. 2, but with varied concentrations of glutamate and malate in the perifusion medium. Higher concentrations of glutamate/malate (5 mM) led only to higher intramitochondrial redox ratios which resulted in higher rates of ketogenesis, a relation found also with isolated mitochondria [21]. Since there was no effect on the rate of respiration, the substrate concentrations in the perifusion medium could be kept lower than those which are usual in incubation experiments.

The results presented in Table 2 were obtained by applying the substrates glutamate/malate and octanoate simultaneously to mitochondria within the permeabilized cells. In order to study the contribution of the different substrates to the hydrogen supply of mitochondria, octanoate, malate and glutamate were added successively to the perifusion medium (Table 3). The respiration rate after permeabilization and in the presence of ADP (octanoate as metabolizable substrate, only) was nearly half of that before permeabilization. The rate of ketogenesis was somewhat lower in permeabilized cells as compared to intact cells. Addition of 1mM malate led to a marked increase in respiration rate (+145%) compared to the respiration rate with octanoate alone. Likewise, the rate of ketogenesis was significantly decreased. Completion of the medium with 1 mM glutamate led to a further small, but significant increase in respiration rate. Furthermore, the redox ratio was significantly increased to values which had been obtained with intact cells before permeabilization. This increased redox ratio was accompanied by a small, but significantly increased rate of ketogenesis. The addi-

Table 4
Respiration rate, intramitochondrial redox ratio (HB/AcAc) and ketogenesis of permeabilized perifused hepatocytes at varied ATP/ADP ratios at the influx site

	Respiration rate (nmol O_2/min per U GLDH)	ATP/ADP_{efflux}	AdN_{efflux} (μmol/l)	HB/AcAc	Ketogenesis (nmol/min per U GLDH)
Before permeabilization					
1 mM Lac, 0.1 mM Pyr, 0.1 mM octanoate, 1 mM Glu, 1 mM Mal	17.0 ± 1.2			2.5 ± 0.4	18.8 ± 0.8
After permeabilization					
+ATP/ADP 0.1	39.4 ± 3.0 *	1.4 ± 0.2	440.4 ± 63.5	2.9 ± 1.7	21.6 ± 8.0
+ATP/ADP 6.7	24.4 ± 0.1 *	5.9 ± 0.5 *	440.4 ± 41.8	2.8 ± 0.4	18.9 ± 0.8
+ATP/ADP 11.6	20.2 ± 1.4 *	6.2 ± 0.7	480.0 ± 10.1	4.4 ± 1.6 *	18.6 ± 4.9
+0.06 mM CAT	5.6 ± 1.4 *			22.8 ± 1.5 *	10.8 ± 4.3 *

Cells were perifused and permeabilized as in Fig. 2 with the exception that the ATP/ADP ratio was varied at the influx site after permeabilization. The sum of adenine nucleotides (AdN) at the influx site was held constant at 500 μmol/l after permeabilization. HB, 3-hydroxybutyrate; AcAc, acetoacetate. Data are given as means ± S.D., $n = 4$.
* $P < 0.05$ (relative to the preceding perifusion conditions, Wilcoxon test).

tion of carboxyatractyloside to the permeabilized cells was followed by reduced respiration rates comparable to the values observed with octanoate as the only substrate. The calculated RCR was about 4, indicating the intactness of oxidative phosphorylation.

The respiration rate depends not only on the source and quantity of reducing equivalents, but also on the energy demand of the cell. To test this on mitochondria in permeabilized cells, the ATP/ADP ratio was varied in the perifusion medium, whereas the sum of adenine nucleotides added was held constant during the experiment (Table 4). Maximum respiration rate was obtained at an ATP/ADP ratio < 1. Increasing this ratio to a value of about 7 at the influx side led to a prompt decrease in respiration rate (from about 40 to about 25 nmol O_2/min per U GLDH, $P < 0.05$). The ATP/ADP ratio at the efflux side did not differ markedly and consistently showed a value of about 6. The HB/AcAc ratio and the rate of ketogenesis were unchanged. An ATP/ADP ratio of about 12 at the influx side led to about half-maximum respiration rate. There was no change in the ATP/ADP ratio at the efflux side, although the HB/AcAc ratio was increased. Only when carboxyatractyloside was added was the resting state obtained. This is shown as a decrease in respiration rate to about 6 nmol O_2/min per U GLDH and an increase in the HB/AcAc ratio to about 23, paralleled by a decrease in ketogenesis to about 11 nmol/min per U GLDH. During the experiment, the sum of the adenine nucleotides (ATP + ADP + AMP) at the efflux side approximately equalled the sum at the influx side, indicating neither an accumulation nor a release of adenine nucleotides from the mitochondria.

4. Discussion

It was the objective of the present work to study some regulatory aspects of energy metabolism of mitochondria within permeabilized hepatocytes. Continuing the perifusion after permeabilizing hepatocytes allowed (1) the establishment of different states of mitochondrial energy metabolism under steady state conditions and (2) the comparison of the metabolic state of mitochondria within permeabilized hepatocytes directly with the metabolic state of the same cells before permeabilization.

Despite the slight mechanical stress caused by perifusion, mitochondria remained within the cells after the digitonin treatment, as has been previously shown [22–24]. The mitochondria can be considered as morphologically intact, since about 90% of activity of GLDH, a sensitive mitochondrial matrix-marker enzyme [15], was present after permeabilization. Taking into account that about 90% of cells originally placed in the perifusion chamber were found at the end of the perifusion period, these cells did not lose any mitochondria during the experiment. Katz and Wals [25] reported that addition of ATP after permeabilization is necessary to maintain the cytoskeleton structure and to retain mitochondria within the permeabilized cells. During our perifusion experiments any lack of ATP was prevented by supplying substrates and ADP to enable ATP production. Microscopic examination of a sample taken from the perifusion chamber revealed that the suspension of permeabilized cells was not contaminated with cellular fragments (not shown). Since almost all cells (90%) were depleted of LDH activity after permeabilization, it is very likely that all low molecular weight substances were also washed out during permeabilization.

In addition to the morphological integrity, the functional intactness of mitochondria after permeabilization was investigated. This had been done by testing mitochondria under the following conditions: (1) variation of sources and concentration of hydrogen supplying substrates; (2) addition of substances affecting mitochondrial energy metabolism such as uncoupler (FCCP) or carboxyatractyloside, an inhibitor of the mitochondrial adenine nucleotide translocase; and (3) the dependence of respiration rate on varied extramitochondrial ATP/ADP ratio. It was demonstrated that all these different states could be investigated within one perifusion experiment (see Fig. 2). Advantageously changing some experimental conditions led to a prompt adjustment of a new steady state of rates of respiration and ketogenesis, and the HB/AcAc ratio. It should be pointed out that the respiration rate with these substrates was stable for 40 min. This is noteworthy, because the substrates used are primarily oxidized by NAD^+-dependent dehydrogenases, which are generally very sensitive to oxidative stress [26]. The influence of lactate/pyruvate on the respiration rate of permeabilized cells should be negligible because more than 90% of LDH activity of the intact cells was lost during permeabilization. On the other hand, respiration rate with glutamate/malate was always somewhat lower compared to what was found with isolated mitochondria [6,7]. This could be due to the presence of $NaHCO_3$ in the perifusion medium leading to partial inhibition of succinate dehydrogenase [27].

The experimental design chosen for this study allowed us to demonstrate some aspects of the interrelation between respiration rate, substrate supply, redox ratio and energy load. This is illustrated in Fig. 3. Taking into account all the different active states (open symbols), the dashed curve represents the dependence of respiration rate on hydrogen supply obtained under different substrate conditions. Octanoate and ADP alone led only to low respiration rates. Adding 1 mM malate successively led to a marked increase in respiration rate and HB/AcAc ratio. Maximum respiration was obtained after a further addition of 1 mM glutamate. Parts of this maximal respiration rate should also originate from an improved fatty acid oxidation because glutamate and malate are intermediates of the citric acid cycle. A further increase of glutamate/malate concentrations up to final concentrations of 5 mM each in the perifusion medium led to a 4-fold increase in HB/AcAc

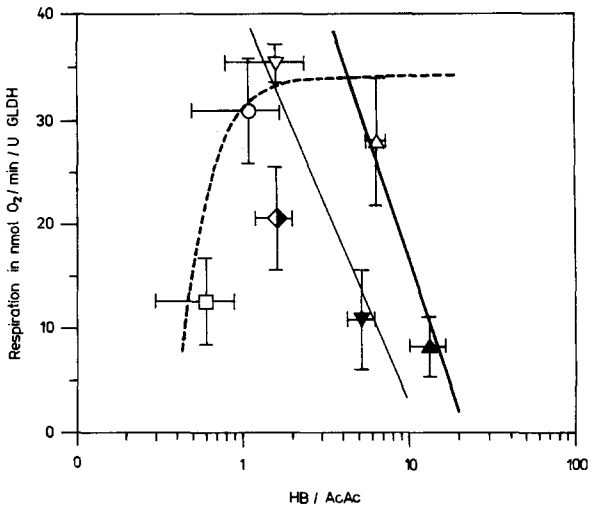

Fig. 3. Relationship between intramitochondrial redox ratio and respiration rate in intact cells and in permeabilized cells at varied concentrations and sources of substrate hydrogen supply. The data points are derived from Tables 2 and 3. Open symbols represent active state respiration (+0.38 mM ADP), closed symbols the resting state (+0.06 mM CAT) for the perifusion with 0.1 mM octanoate (□), 0.1 mM octanoate plus 1 mM malate (○), 0.1 mM octanoate plus 1 mM malate plus 1 mM glutamate (▽,▼), or 0.1 mM octanoate plus 5 mM malate plus 5 mM glutamate (△,▲). Data for intact cells before permeabilization under gluconeogenic conditions in the presence of 0.1 mM octanoate, 1 mM lactate, and 0.1 mM pyruvate are given by (◇). The dashed line represents the active state of mitochondria in permeabilized cells. The straight lines are regression lines calculated from the corresponding single data points of the active (▽,△) and resting state (▼,▲).

ratio whereas the respiration rate was unchanged. This could be an indication that under these conditions the respiratory chain is limiting oxidative phosphorylation. So, maximal respiration rates have already been obtained with the lower glutamate/malate concentrations (1 mM each) which correspond to the cytosolic levels in intact hepatocytes [28,29]. On the other hand, mitochondria within permeabilized cells responded to increasing extramitochondrial ATP/ADP ratios with decreased respiration as demonstrated by the data in Table 4. The resulting relation between respiration and redox state depends on the hydrogen supply. If the phosphorylation of ADP was inhibited by carboxyatractyloside so that the mitochondria were in a resting state, an improved hydrogen supply resulted in higher redox ratios. The shift in the relation between respiration and the redox ratio which is produced by a transition from the active to the resting state is indicated by the straight lines in Fig. 3. It should be mentioned that the data in Table 4 are close to the straight line in Fig. 3.

Advantageously, our experimental approach enabled us directly to compare parameters of the energy metabolism of intact and permeabilized hepatocytes. Although the medium chosen for perifusion is unusual for studies on intact hepatocytes (lower sodium and higher potassium content as well as some mannitol which can permeate the plasma membrane but not the mitochondrial inner membrane [30]) rates of respiration and gluconeogenesis (not shown) were in the same range as those which have been found by others [12]. Looking at respiration rates and redox ratios before permeabilization, mitochondria under these in situ gluconeogenic conditions (lactate, pyruvate and octanoate as substrates) seem to be in an intermediate state of respiration. The in situ point in Fig. 3 is characterized by a non-optimal supply with reducing equivalents enabling only about 60% of maximal respiration. On the other hand, mitochondria in situ do not work in an active state under this limited substrate supply since the redox ratio was significantly higher than the ratio which would be expected for the active respiration of this magnitude (see the dashed line in Fig. 3). Therefore, it is concluded that respiration is limited by the substrate supply as well as by ADP phosphorylation reaching only about 50% of the span between resting and active respiration. These findings are in line with results demonstrating the remarkable stimulation of respiration in hepatocytes under gluconeogenic conditions by uncoupling [2]. Furthermore, it has been reported that the increase in respiration of hepatocytes after stimulation with gluconeogenic precursors or Ca^{2+}-releasing hormones is not followed by significant changes in adenine nucleotide patterns [1,2]. These results can be explained by the relations demonstrated in Fig. 3. An improved hydrogen supply by gluconeogenic substrates [2] or via Ca^{2+}-dependent dehydrogenases [3,4] can compensate for the expected decrease in the ATP/ADP ratio which would result from an increased ATP demand at constant hydrogen supply.

In conclusion, mitochondria in permeabilized perifused hepatocytes remain morphologically and functionally stable at 37°C for as long as 40 min. The described method simplifies the preparation of permeabilized cells and provides a means to study functions of mitochondria under such metabolic states which are adapted to that within the intact cell. It offers possibilities to study phenomena of metabolic short-term regulation as well as the effects of hormones or pharmaceutical drugs on metabolic pathways within different structural levels of the cell under real stationary conditions.

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