ON THE OXIDATION OF SUCCINATE BY PARENCHYMAL CELLS ISOLATED FROM RAT LIVER

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1. Introduction

There have been several reports [1–3] that succinate increases the oxygen consumption of isolated parenchymal cells prepared from rat liver. Succinate has also been reported to support energy-dependent Ca^{2+} accumulation by liver cells [3]. Yet, with the perfused liver, succinate and other citric acid cycle intermediates will not support gluconeogenesis [4] because of permeability barriers [5]. We considered several possible explanations for the apparent oxidation of succinate by isolated liver cells. These include the possibility that liver cells are more permeable to citric acid cycle intermediates than perfused liver and the possibility that succinate is oxidized only by damaged cells.

The liver cells used in this investigation appeared intact (93 ± 2% excluded trypan blue) and gave excellent rates of gluconeogenesis from lactate (1.2 μmol/min/g wet weight). However, the results indicate that the more than two-fold increase in oxygen consumption over control rates caused by succinated is primarily due to the oxidation of succinate to malate by damaged cells.

2. Materials and methods

Isolated hepatic cells were prepared from 48 hr starved male Wistar rats (180–240 g) by the method of Berry and Friend [6] with the modifications of Cornell et al. [7] and Krebs et al. [8]. The isolated cells were washed three times and suspended in Krebs-Henseleit saline. The dry weight of the cells was multiplied by a factor of 3.7 [7] to obtain wet weight.

Incubation were carried out in Warburg flasks for 60 min at 37°C with an atmosphere of 95% O_2 and 5% CO_2. To maintain the 5% CO_2 atmosphere, the center wells and both side arms of the flasks contained a mixture 2.25 M in $KHCO_3$ and 0.75 M in K_2CO_3 with 5 mg of carbonic anhydrase in a total volume of 2 ml [8]. Both side arms and the center well contained the bicarbonate–carbonate solution to provide a large surface area for gas exchange. Incubations were carried out with 60–80 mg wet weight of cells in a final volume of 4 ml of Krebs-Henseleit saline supplemented with 2.5% albumin (charcoal treated and dialyzed; Fraction V, Sigma Chemical Co.). Oxygen uptake was determined manometrically at 5 min intervals between 20 and 60 min of incubation. Glucose and malate were measured on KOH-neutralized $HClO_4$ extracts by standard enzymatic methods described in Bergmeyer [9]. Cell viability was determined by the criteria of trypan blue exclusion [10] with a microscope, hemacytometer counting chamber, and a hand tally counter. Radioactive glucose was isolated by the procedure outlined by Blair et al. [11] and counted by liquid scintillation.

Intentionally damaged cells were obtained by adding 2% digitonin until all of the cells of the preparation were permeable to trypan blue (final concentration, 0.001% digitonin).

In some experiments, the cells were separated from the incubation medium by a special 'hepatocyte-separation tube' developed in the laboratory of H. A. Krebs by R. Hems and P. Lund. It is constructed of glass and consists of a bulb containing 6% $HClO_4$ in 4%

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NaCl below a compartment containing the incubation medium and cells separated by a capillary tube filled with 4% NaCl. Centrifugation of the tubes in a clinical centrifuge separates the cells rapidly and cleanly from the incubation medium.

3. Results and discussion

The results of table 1 show that incubation of the isolated liver cells used in these experiments with lactate as substrate gave acceptable rates of gluconeogenesis (1.2 μmol/min/g wet weight), that the cells were of good viability (93% excluded trypan blue), and that the cells gave reasonable P/O ratios (2.0). The rates of gluconeogenesis found in this study with lactate (plus lysine) agree well with rates reported by other laboratories with either perfused liver or isolated liver cells. Indeed, Cornell et al. [12] reported almost identical rates to those shown in table 1 with lactate (plus lysine).

Trypan blue exclusion is often used as an index of viability of isolated liver cell preparations with most laboratories reporting viabilities of 90 to 95%, as found in the present study.

The P/O ratios reported in table 1 were calculated from the 'extra' oxygen consumption and the known ATP requirement for glucose synthesis from either lactate or succinate. The P/O ratios calculated with lactate (plus lysine) as substrate are lower than the P/O ratios reported by Williamson et al. [13] with perfused liver but agree well with the P/O ratios which can be calculated from the data of Krebs et al. [8] with isolated liver cells.

Thus, it would appear that the isolated cells used in this study were as intact and viable as cell preparations routinely used in other laboratories. However, as reported by others [1–3], succinate was found to produce a substantial increase in oxygen consumption over respiration with endogenous substrates (table 1). Although this increase was even greater than that caused by lactate, glucose was synthesized at a much slower rate with succinate as substrate. Therefore, the P/O ratio calculated with succinate as substrate was much lower than with lactate (table 1). When succinate and lactate were combined, the increased oxygen consumption ($\triangle O_2$) and increased glucose synthesis were almost equal to the sum of the increased oxygen consumption and glucose synthesis when these substrates were added separately. This additive effect suggests that succinate did not affect the metabolism of lactate and that lactate did not affect the metabolism of succinate by the cells. The dilemma then was why succinate increased oxygen consumption

Table 1
Oxygen consumption and glucose production from lactate and succinate by isolated parenchymal cells

Viability	Additions	O_2 Consumption	$\triangle O_2$ Consumption	Glucose Production	Malate Production	P/O Ratio
%	(mM)	μmol/min/g wet weight				
93 ± 2	None	2.7 ± 0.1	——	0.09 ± 0.01	0.00 ± 0.01	——
93 ± 2	Lactate (10)	4.3 ± 0.1	1.6	1.18 ± 0.02	0.01 ± 0.01	2.0 ± 0.1
93 ± 2	Succinate (20)	5.1 ± 0.2	2.4	0.21 ± 0.01	3.1 ± 0.1	0.10 ± 0.02
93 ± 2	Lactate (10) + succinate (20)	6.6 ± 0.2	3.9	1.31 ± 0.01	3.3 ± 0.02	0.9 ± 0.1
0.0	Digitonin	<0.02	——	0.04 ± 0.02	0.01 ± 0.01	——
0.0	Digitonin + succinate (20)	12.7 ± 0.6	12.7	0.08 ± 0.03	19.7 ± 1.5	0.01 ± 0.01

The incubation medium used contained 2mM lysine and 60–80 mg wet weight of cells. The results are presented as the means ± SEM for four preparations of liver cells. Viability was based on exclusion of trypan blue. The P/O ratios with lactate were calculated from 3 times the increase in the rate of glucose production divided by the increase in rate of oxygen consumption. With succinate as substrate the equation used was 2 times the increase in rate of glucose production divided by the increase in oxygen consumption. With digitonin it was necessary to reduce the amount of cells incubated to about 10 mg wet weight of cells to maintain nearly linear rates of oxygen consumption and malate production.

and yet failed to serve as a substrate for gluconeogenesis. With succinate (plus rotenone) as substrate, Berry [1] observed but did not elaborate upon an accumulation of malate by isolated liver cells. This observation was followed up and, as shown in table 1, malate was found to accumulate at a very rapid rate with succinate as substrate. In contrast, essentially no malate accumulated with lactate as substrate. Indeed, the rate at which malate accumulated in the presence of succinate accounted for the bulk of the increased rate of oxygen consumption observed with succinate as substrate.

The question why malate accumulated from succinate oxidation with essentially no synthesis of glucose was investigated by determining the distribution of malate between the incubation medium and the cells with the hepatocyte separation tube. In three different cell preparations incubated with 20 mM succinate for 60 min, more than 99% of the accumulated malate was found outside of the cells. The question then was whether it was characteristic of the liver to take up succinate and release malate. This possibility was eliminated with the use of the isolated perfused liver preparation perfused for 120 min with Krebs-Henseleit saline fortified with 2.5% serum albumin. In contrast to the results obtained with isolated liver cells, essentially no conversion of exogenous succinate (20mM) to malate was observed with perfused livers. This experiment demonstrated that it is not characteristic of liver to take up succinate and release malate. However, a more definitive experiment on this point was conducted with the isolated liver cells. It has been shown that intramitochondrial malate is an obligatory intermediate in the synthesis of glucose from pyruvate [14,15]. Therefore, if succinate enters viable, glucose synthesizing cells the formation of malate in the mitochondria would dilute the labeled malate pool produced from radioactive pyruvate. Dilution of the labeled malate pool would decrease the incorporation of labeled pyruvate into glucose. It was found, however, that succinate decreased neither the specific activity nor the amount of labeled glucose formed from [1-^{14}C]-pyruvate (10 mM) by isolated liver cells.

It was then predicted that if 'damaged' or broken cells of these reasonably intact cell preparations were primarily responsible for the oxidation of succinate, then deliberately damaged preparations of cells should convert succinate to malate at even greater rates. Digitonin at a final concentration of 0.001% was found to render all cells permeable to trypan blue (table 1). Endogenous respiration by the cells was abolished by digitonin but succinate oxidation to malate was increased five-fold (table 1). Thus, it has to be concluded that succinate oxidation is primarily a property of the damaged cells of a liver cell preparation.

The capacity of a liver cell preparation to oxidize exogenous succinate would seem to provide an excellent means for determining the relative intactness of liver cell preparations. In the present study the percent viability would be 84% when calculated from the rate of malate accumulation with the intact cells versus the rate with digitonin disrupted cells. Also, from oxygen consumption studies (table 1) the viability would be calculated to be 81% based on the amount of increase over the control ($\triangle O_2$) brought about by succinate in the intact cells versus that of the digitonin treated cells. The reason(s) for the apparent discrepancy between these estimates of viability and the trypan blue estimate (93%) is not known. However, one contribution factor may be that succinate was found to induce a slight but nevertheless significant increase ($P<0.001$) in the rate of gluconeogenesis (table 1). This suggests that succinate may very slowly penetrate those cells of the preparation with the capacity to synthesize glucose. However, it can be calculated that only about 5% of the increased oxygen consumption caused by succinate could be explained on this basis.

The cell preparations used in this study were also examined for the capacity to oxidize and to use other citric acid cycle intermediates for gluconeogenesis. It was found that citrate, isocitrate, α-ketoglutarate, fumarate and malate would not increase oxygen consumption nor function as effective substrates for gluconeogenesis. These compounds would not be expected to penetrate intact cells [5] but would be expected readily to penetrate damaged cells. However, because of the Ca^{2+} content of Krebs-Henseleit saline, the mitochondria of damaged cells would also be extensively damaged. On the basis that damaged mitochondria are known to lose that capacity to oxidize NAD^+-linked substrates without losing the capacity to oxidize succinate, the results obtained with these citric acid cycle intermediates are readily rationalized. On the other hand, it was found that oxaloacetate accelerated oxygen consumption by

liver cells. However, in contrast to succinate, oxaloacetate supported a rapid rate of gluconeogenesis. These results can be explained on the basis that oxaloacetate is unstable and decarboxylates to pyruvate, a good substrate for gluconeogenesis by liver tissue.

This study demonstrates that the oxidation of succinate by liver cells is carried out primarily by the damaged cells of liver preparations. This study should serve to caution other investigators against the use of succinate as a source of either carbon or energy for metabolic studies with isolated liver cells. Likewise, it is made apparent in this study that even a small percentage of damaged cells represents a potential source of artifacts in metabolic studies with isolated parenchymal cells.

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