Mitochondrial compartmentation of metabolic CO_2 resulting from its site of origin in relation to urea synthesis

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In isolated hepatocytes the entry into urea of metabolic $^{14}CO_2$ derived from [^{14}C] formate is modified by the addition of dichloroacetate and hydroxypyruvate. An explanation is that this results from changes in the cytoplasmic/mitochondrial pH gradient. $^{14}CO_2$ derived from [1-^{14}C]alanine enters into urea more readily than $^{14}CO_2$ arising from [1-^{14}C]glutamate. It is proposed that the difference, which is more than 4-fold, is indicative of a preferred pathway for metabolic CO_2 in liver mitochondria from pyruvate dehydrogenase to carbamoylphosphate synthetase than form oxoglutarate dehydrogenase. Acetazolamide inhibition of carbonic anhydrase is without effect on this observed incorporation into urea.

Hepatocyte *Mitochondria* *Urea synthesis* *Enzyme location* *CO_2 compartmentation* *Cytosolic/mitochondrial pH gradient*

1. INTRODUCTION

In isolated hepatocytes, metabolic CO_2 has been shown to enter into urea more readily than the exogeneous bicarbonate plus CO_2 from the suspending medium [1]. Substrates which, when metabolised, affect the pH of the cell (dichloroacetate and hydroxypyruvate) have been shown to modify the fixation of $^{14}CO_2$ into urea [2]. The direction of the change in specific activity of urea, positive or negative, depends on two factors. First, the direction of the shift in intracellular pH which the modifiers cause, and second, the site of origin (extracellular or intracellular) of the $H^{14}CO_3$ fixed into carbamoylphosphate. Formate is metabolised in isolated hepatocytes by the formation of the tetrahydrofolate compound and through the NADP-linked formyltetrahydrofolate, dehydrogenase CO_2 is formed in the cytosol [3,4]. The effect of dichloroacetate and hydroxypyruvate on the incorporation into urea of cytosolic $^{14}CO_2$ from ^{14}C formate has been examined.

When [1-^{14}C]alanine is oxidized in isolated hepatocytes, a principle site of $^{14}CO_2$ production is pyruvate dehydrogenase (PDH). This is indicated by the increase of more than 100% in the formation of $^{14}CO_2$ from [1-^{14}C]alanine on addition of dichloroacetate. When [1-^{14}C]glutamate is the substrate, the site of formation of $^{14}CO_2$ is oxoglutarate dehydrogenase. These enzymes are located in the mitochondrial matrix. A study in hepatocytes from 48-h starved rats of the relative rates of incorporation of $^{14}CO_2$ derived from these two substrates reveals that CO_2 from alanine enters into urea with greater facility than CO_2 derived from glutamate.

2. MATERIALS AND METHODS

Isolated hepatocytes were prepared from 48-h starved rats of the Wistar strain as in [5] with the modifications described in [6]. The method of incubation, collection of $^{14}CO_2$, and measurement of the specific activity of urea was as in [1,2]. Radioactive substrates were obtained from Amersham International (Amersham, Bucks). The ethanol which they contained was removed by rotary evaporation to dryness and the residue was

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redissolved in water. Dichloroacetic acid (DCA), hydroxypyruvate and urease grade V were supplied by Sigma (Poole, Dorset). Enzymes and coenzymes were obtained from Boehringer (Lewes, East Sussex).

3. RESULTS

3.1. *Effect of dichloroacetate and hydroxypyruvate on the incorporation of cytoplasmic CO_2 into urea*

The results in table 1, experiment 1, show that dichloroacetate markedly changes the specific activity of urea and that the magnitude of the change depends on the site of origin of the $H^{14}CO_3^-$ used in the carbamoylphosphate synthetase reaction. When the site of formation was mitochondrial (flasks 1 and 2 contained [1-^{14}C]alanine), the effect of dichloroacetate was to lower the specific activity of urea by more than 40%. Because dichloroacetate stimulated the production of labelled CO_2 by over 100%, the overall effect of DCA on the entry of mitochondrial metabolic CO_2 into urea (corrected for a zero change in labelled CO_2 production) may be as great as −150%. An explanation of this result is that DCA causes a rapid efflux of metabolic CO_2 from mitochondria. In contrast, the effect of DCA on the incorporation into urea of CO_2 formed in the cytosol from formate was much smaller. However, cytosolic CO_2 must enter the mitochondria in order to be incorporated into urea, and in the face of an increase in the efflux of CO_2 from mitochondria a decrease in the incorporation of cytosolic CO_2 is to be expected.

Table 1

Effect of dichloroacetate and hydroxypyruvate on the entry of ^{14}C into urea from [1-^{14}C]alanine and [^{14}C]formate in isolated hepatocytes from 48-h starved rats

	Radioactivity in CO_2 (dpm/flask $\times 10^{-3}$)	Percent change in dpm in CO_2	Radioactivity in urea (dpm/flask $\times 10^{-3}$)	Urea formed (μmol/flask)	Specific activity in urea (dpm $\times 10^{-3}$/μmol)	Percent change in specific activity of urea	Percent change in specific activity of urea corrected for zero change in CO_2
Exp. 1 plus dichloroacetate							
1	91.7		6.5	1.27	5.1		
2	186.6	+103.5±16.7 (3)	4.1	1.44	28.5	−44.3± 7.6 (3)	−147.8±24.1 (3)
3	1161.5		19.7	1.35	14.6		
4	1119.9	− 3.6± 5.6 (3)	19.7	1.46	13.5	− 6.8± 4.8 (3)	− 3.2± 9.2 (3)[a]
Exp. 2 plus hyroxypyruvate							
1	84.4		10.1	2.33	4.33		
2	54.3	− 35.7± 3.3 (3)	18.7	1.84	10.16	+134.7±51.9 (3)	+170.4±54.6 (3)
3	458.8		13.2	2.41	5.46		
4	560.7	+ 22.2±14.6 (3)	15.1	1.96	7.69	+40.7±27.9 (3)	+ 18.5±14.0 (3)[b]

Flasks 1 and 2 contained 5 mM [1-^{14}C]alanine, 2 mM formate, 0.5 mM methionine. Flasks 3 and 4 contained 5 mM alanine, 2 mM [^{14}C]formate and 0.5 mM methionine. Dichloroacetate (2 mM) or hydroxypyruvate (2 mM) was added to flasks 2 and 4 as shown. Each flask contained a 4-ml suspension of hepatocytes in Krebs-Henseleit saline [10]. The mean wet weight of cells was 88.2±16.2 mg SD (6). Flasks were incubated in duplicate for 30 min at 38°C. The values for radioactivity and urea are means of 3 experiments. Because of variation in cell content of individual experiments, statistics are presented only on the percentage changes. *P* values ([a] < 0.0006; [b] < 0.009) are calculated for the effects of origin of the metabolic CO_2

In experiment 2, table 1, the addition of hydroxypyruvate had the opposite effect to dichloroacetate. The efflux of CO_2 from mitochondria was decreased, thereby preserving CO_2 for fixation into urea. The increase in specific activity of urea which resulted from mitochondrial $^{14}CO_2$ (corrected for zero change in CO_2) was about 170%. In the case of cytoplasmic CO_2 the increase was much smaller (18.5% corrected value).

Our previous proposal was that dichloroacetate and hydroxypyruvate, when metabolised, resulted in an increase or decrease in the cytoplasmic/mitochondrial pH gradient and that this affects the incorporation of both intracellular and extracellular bicarbonate plus CO_2 into urea in opposite directions. The results obtained with [^{14}C]formate indicate more specifically that this applies to cytosolic, rather than extracellular CO_2.

3.2. *Dependence on the site of origin of mitochondrial metabolic CO_2 on its incorporation into urea*

Table 2 presents results which show that the proportion of metabolic $^{14}CO_2$ resulting from the oxidation of [1-^{14}C]alanine or [1-^{14}]glutamate entering into urea may differ by a factor of more than four.

In flasks 1–4 the main source of both urea carbon and urea nitrogen is alanine, only the source of metabolic $^{14}CO_2$ varies. In flasks 5 and 6 the main supply of nitrogen is NH_4Cl while the carbon for urea is derived mainly from asparagine. The $^{14}CO_2$ which is incorporated into urea is derived either from 'carrier free' [1-^{14}C]alanine or [1-^{14}C]-glutamate. In both cases the concentration of the labelled substrate wa 0.01 mM. A comparison of the proportion of $^{14}CO_2$ entering urea to the total metabolic $^{14}CO_2$ formed shows that CO_2 derived

Table 2

Effect of acetazolamide and substrate concentration on the incorporation of ^{14}C into urea from [1-^{14}C]glutamate

Flask no.	1	2	3	4	5	6
Total radioactivity in flask (dpm × 10^{-4})	49.2 ± 0.59	49.6 ± 0.48	49.9 ± 0.19	51.1 ± 2.0	23.7 ± 0.65	49.9 ± 0.54
Radioactivity in CO_2 + HCO_3^- (dpm × 10^{-4}/flask)	3.7 ± 0.79	3.5 ± 0.44	12.2 ± 1.8	11.7 ± 2.6	5.8 ± 1.5	13.0 ± 1.1
Specific activity of CO_2 + HCO_3^- (dpm × 10^{-2} μmol)	2.44 ± 0.38	2.31 ± 0.22	8.08 ± 0.11	7.82 ± 0.16	3.82 ± 0.74	8.65 ± 0.74
Urea found (μmol/flask)	1.58 ± 0.41	1.25 ± 0.34	1.54 ± 0.46	1.21 ± 0.33	1.58 ± 0.51	1.55 ± 0.61
Radioactivity in urea (dpm × 10^{-2}/flask	25.4 ± 12.9	21.2 ± 11.1	21.0 ± 7.4	20.1 ± 9.0	48.9 ± 28.5	52.4 ± 21.0
Specific activity of urea dpm × 10^{-2}/μmol)	15.09 ± 0.36	16.03 ± 0.44	13.99 ± 0.47	16.05 ± 0.59	29.77 ± 0.98	38.47 ± 2.0
Ratio $\frac{\text{specific activity urea}}{\text{specific activity } CO_2 + HCO_3^-}$	6.11 ± 0.50	6.86 ± 1.3	1.32 ± 0.14[a]	1.47 ± 0.26[a]	8.47 ± 3.6	3.14 ± 0.62[b]

Each flask contained a 4-ml suspension of isolated hepatocytes from 48-h starved rats. The mean weight of cells was 85.06 ± 9.0 SD per flask. Flasks 1 and 2 contained 5 mM [1-^{14}C]alanine; flasks 3 and 4 contained 5 mM alanine and 0.2 μCi [1-^{14}C]glutamate 'carrier-free'. Acetazolamide (0.5 mM) was added to flasks 2 and 4. Flasks 5 and 6 contained 1 mM asparagine, 2 mM NH_4Cl and 0.5 mM ornithine. In addition, flask 5 contained 0.1 μCi [1-^{14}C]alanine and flask 6, 0.2 μCi [1-^{14}C]glutamate, both 'carrier-free'. Flasks were incubated for 30 min at 38°C in duplicate. Values are means ± SD for 3 experiments. *P* values are calculated for the differences in ratios between [1-^{14}C]alanine and [1-^{14}C]glutamate-derived urea: [a] < 0.002; [b] < 0.006. The specific activity of CO_2 + HCO_3^- is based on the acid volatile CO_2 found of 144.6 μmol ± 7.9 SD (42) per flask. When carrier-free substrates were used, without additional substrate, the final concentration was 0.01 mM. Ethanol added as a preservative to radiochemicals was removed by rotary evaporation

from [1-^{14}C]alanine enters into urea with greater facility than CO_2 derived from [1-^{14}C]glutamate. The differences are up to 4.5-fold in favour of alanine, and are independent of the origin of the urea nitrogen.

Acetazolamide is without effect on the incorporation of radioactivity into urea. There is some indication of a decrease in the synthesis of urea from alanine (1.56 μM/flask to 1.23 μM) in the presence of acetazolamide (means of 6 values) but this is not statistically significant. The results show that, independent of the origin of the nitrogen of urea, metabolic mitochondrial CO_2 does not enter into carbamoylphosphate synthesis equally from all the sites of formation.

4. DISCUSSION

The work in [7] has drawn attention to the extent to which metabolic CO_2 may undergo fixation into intermediate products without equilibration with the general pool of bicarbonate plus CO_2. Further work [1] confirmed this by showing that metabolic CO_2 enters into urea synthesis more readily than it exchanges with the medium bicarbonate plus CO_2, and the CO_2 of the gas phase. Later experiments [2] showed that dichloroacetate and hydroxypyruvate alter the relative incorporation into urea of $^{14}CO_2$, generated in the mitochondria from [1-^{14}C]alanine or from $H^{14}CO_3^-$ added to the medium. The direction in which these changes occur depends on the site of origin of the incorporated label. It was concluded that the effects resulted from opposing shifts in the pH of the extracellular or mitochondrial compartments.

Here, $^{14}CO_2$ generated in the cytosolic compartment from [^{14}C]formate behaved in a similar manner to the $H^{14}CO_3^-$ added to the medium. It is concluded that a more precise statement of the effect is that dichloroacetate and hydroxypyruvate alter the mitochondrial/cytosolic pH gradient in opposite directions resulting in the loss or retention of labelled CO_2 at the site of fixation, as HCO_3^-, into carbamoylphosphate. Furthermore, the difference in the observed ratios of specific activity of urea to specific activity of total bicarbonate, which results from mitochondrial or cytosolic metabolic CO_2 (9.54 and 18.5, respectively) is indicative of mitochondrial/cytosolic compartmentation of CO_2. This is contrary to the statement in [8]. However, in that work the period of incubation was 1 h. In earlier work from this laboratory we noted that the effect was greater in the early part of the time course, and as expected the obervable effects declined as the label became distributed throughout the total bicarbonate pool.

Acetazolamide, a specific inhibitor of carbonic anhydrase, has been shown to inhibit citrulline synthesis in isolated mitochondria from guinea pig liver at concentrations of about 50 μM [8]. In the present experiments, using isolated rat hepatocytes, acetazolamide at a concentration of 500 μM, depressed urea synthesis from alanine by only 20% and this value was not statistically significant. Similarly, the entry into urea of metabolic $^{14}CO_2$ was not significantly affected. In [9] it was shown that 50 μM acetazolamide inhibits gluconeogenesis and ureogenesis in isolated hepatocytes only in a medium low in bicarbonate. In Krebs-Henseleit bicarbonate saline [10] the effect of acetazolamide was marginal.

Evidence of microcompartmentation or substrate channelling in mitochondria has been reported in [11] and [12]. The latter defined microcompartmentation as a situation in which a metabolite appears to move from an enzyme by which it is synthesized to a second enzyme for which it is a substrate without first mixing with the total metabolite pool in the same compartment. This definition may be extended to include a situation where a metabolite which is the product of more than one enzyme in the same compartment is used by another enzyme more readily from one source than from the other.

Authors in [11] considered the several possibilities which may account for apparent compartmentation including the existence of separate metabolite pools, heterogeneity of isolated liver cells with differing metabolic roles or the possibility of different mitochondrial types.

The author in [13] has discussed at length the evidence for a spatial arrangement of enzymes into microenvironments in which, because of the proximity of enzymes of production and utilisation, channelling of a metabolite may occur.

From these present findings one may conclude that, assuming that the Krebs cycle enzymes are organized in a multi-enzyme complex, oxoglutarate dehydrogenase is placed in a less advantageous position as a supplier of CO_2 to car-

bamoylphosphate synthetase, than pyruvate dehydrogenase. The multiple role which pyruvate dehydrogenase plays might be expected to place it outside of a Krebs cycle enzyme microenvironment, as proposed in [13].

The ready accessibility of CO_2 from pyruvate dehydrogenase to carbamoylphosphate synthetase and the comparative inaccessibility of CO_2 from oxoglutarate dehydrogenase is indicative of some form of compartmentation of CO_2 within the liver mitochondria, in spite of the free diffusion of CO_2 [14]. It is possible that the hydration of CO_2 occurs so close to the site of formation that the differences which are reported must be considered in terms of the less diffusible bicarbonate ion [14].

Heterogeneous distribution of glutamine synthetase in rat liver parenchymal cells has been reported in [15] where it was shown that this enzyme is found in only 7–8% of the cell population. In [16] it was found that carbamoylphosphate synthetase has a distribution complementary to that of glutamine synthetase which places it in the majority of the parenchymal cells.

Authors in [17] have calculated that carbamoylphosphate synthetase (ammonia) may constitute between 22–26% of the matrix protein and that, based on the daily urea output of the adult rat, on average only about 8% of the capacity of this enzyme is used. In the experiments reported here the rates of urea synthesis are sufficiently low as to leave considerable spare capacity. In view of this, it is perhaps surprising that there are such marked differences in the utilization of metabolic CO_2, and it serves to strengthen the conclusion that the oxoglutarate dehydrogenase is isolated in a microenvironment.

For cell heterogeneity to play a role in the present findings, some form of separation of the carbamoylphosphate synthetase containing cells on the basis of oxoglutarate or alanine oxidation would be necessary and this seems very unlikely. Similar conditions would apply in the consideration of mitochondrial heterogeneity. However, if mitochondrial carbonic anhydrase is implicated in carbamoylphosphate synthesis as proposed in [8], its location adjacent to pyruvate dehydrogenase, and distant from oxoglutarate dehydrogenase, could account for the present observation. Failure to find an effect of acetazolamide does not support this proposition.

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