Quantifying the Carboxylation of Pyruvate in Pancreatic Islets*

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Pyruvate has been estimated to enter the citric acid cycle in islets by carboxylation to the same extent or more than by decarboxylation. Those estimates were made assuming the dimethyl esters of [1,4-14C]succinate and [2-3-14C]succinate, incubated with islets at a concentration of 10 mM, gave the same ratio of 14CO2 yields as if [1-14C]acetate and [2-14C]acetate had been incubated. The labeled succinates, at 10 mM, but not 1 mM, are now shown to give ratios higher than the labeled acetates at those concentrations and therefore higher estimates when related to yields from [2-14C]glucose and [6-14C]glucose. Using the labeled acetate ratios in paired incubations, the rate of pyruvate carboxylation is still estimated to be about two-thirds the rate of pyruvate decarboxylation. Participation of the malic enzyme-catalyzed reaction explains the greater ratio of yields of 14CO2 from the succinates at 10 mM than 1 mM and increases in those ratios on glucose addition and can account for the removal from the citric acid cycle of oxaloacetate carbon formed in the carboxylation.

Pyruvate carboxylation to oxaloacetate on incubation of islets with glucose has been estimated to occur to as great an extent or more than pyruvate decarboxylation (1–3). If correct, that has important implications with regard to islet and presumably β cell metabolism. Since in the turning of the citric acid cycle the amount of oxaloacetate is unchanged, i.e. oxaloacetate + acetyl-CoA → citrate → 2CO2 + oxaloacetate, at least half the carbon of glucose entering the citric acid cycle would have to leave the cycle in a product or products other than CO2. The formation of so much oxaloacetate and hence its product(s) would presumably have a purpose.

The estimates depend in principle upon a comparison of the ratio of the yields of 13CO2 from [2-14C]pyruvate and [3-14C]pyruvate to those from [1-14C]acetate and [2-14C]acetate (4). Since [2-14C]pyruvate and [3-14C]pyruvate on decarboxylation yield [1-14C]acetate and [2-14C]acetate, respectively, if pyruvate's metabolism were only via decarboxylation to acetyl-CoA, the ratios from the labeled pyruvates and acetates should be the same (Fig. 1). Since [3-14C]acetate is oxidized to 12CO2 in fewer turns of the citric acid cycle than [2-14C]acetate, unless the only fate of the carbons of acetate is to CO2, the yield of 12CO2 from [1-14C]acetate will exceed that from [2-14C]acetate. If pyruvate is carboxylated because of rapid equilibration between oxaloacetate and fumarate, both labeled pyruvates should yield [2,3-14C]oxaloacetate (Fig. 1). If equilibration is complete, the yield of 14CO2 from both pyruvates would be the same. Therefore, to the extent there is carboxylation relative to decarboxylation, the ratio of the yields from the labeled acetates should exceed that from the labeled pyruvates.

MacDonald (2, 3) substituted the yields of 12CO2 from [2-14C]glucose and [6-14C]glucose for those from the labeled pyruvates, since [2-14C]pyruvate and [3-14C]pyruvate are formed, respectively, via the Embden-Meyerhof pathway from those labeled gluoses. MacDonald substituted the yields of 12CO2 from [1,4-14C]succinate and [2,3-14C]succinate for those from the labeled acetates, since the labeled succinates are formed, respectively, in the initial turn of the citric acid cycle from [1-14C]acetate and [2-14C]acetate (4). Actually, the dimethyl esters of the labeled succinates were used, since the esters penetrate the cell membrane and once inside the cell are hydrolyzed. Ratios of yields of 12CO2 from the labeled gluoses were about 2. Ratios of 4–6 or more, the larger in the presence of unlabeled gluose, were observed when the labeled succinates were incubated at 10 mM concentration, resulting in the quantitation of equal or greater rates of pyruvate carboxylation than decarboxylation.

Uncertainty exists with regard to the quantitation. First, the method of Kelleher and Bryan (4) specifies the use of the paired flask technique, i.e. labeled pyruvate in the presence of unlabeled acetate or equivalent and labeled acetate in the presence of unlabeled pyruvate or equivalent incubated under identical conditions. Second, the metabolism of acetate and succinate are not equivalent, even though the fate of acetate and succinate tracers will be identical if incubated under identical conditions. In the citric acid cycle a quantity of acetate, but not succinate, is oxidized to CO2, i.e. succinate → oxaloacetate + acetyl-CoA → citrate → 2CO2 + succinate. We have now incubated the 13C-labeled gluoses, acetates and dimethyl succinates with islets using the paired flask technique and from the ratios of the yields of 12CO2 quantified pyruvate carboxylation.

EXPERIMENTAL PROCEDURES

Materials—D-[2-14C]Glucose, 5.5 mCi/mM, reported to be >99% pure, was purchased from American Radiolabeled Chemicals, St. Louis, MO. On high pressure liquid chromatography using an HPX-87P column (Bio-Rad) with water at 80 °C as solvent, it gave a single peak with the mobility of glucose and over 99% of the 14C. D-[6-14C]Glucose, 4.9 mCi/mM, sodium [1-14C]acetate, 56.8 mCi/mM, and sodium [2-14C]acetate, 56.0 mCi/mM, were purchased from DuPont NEN. The [1-14C]acetate and [2-14C]acetate were received dissolved in ethanol, which was evaporated before use. Dimethyl [1,4-14C]succinate, 11 mCi/mM, and dimethyl [2,3-14C]succinate, 8 mCi/mM, each reported to be >98% pure, and unlabeled dimethyl succinate were purchased from Sigma.

Islets—Islets were isolated from pancreata of fed 200–300-g male Sprague-Dawley rats. The pancreata were digested with collagenase, and islets in the digest were collected under stereomicroscopy using a glass pipette. Unless otherwise noted, the islets were cultured for 20 h in RPMI 1640 medium, 11 mM glucose, 10% fetal calf serum, 100 units of penicillin G/mL, and 100 μg/mL streptomycin/mL. They were preincu-
bated for 30 min in 3.3 mM glucose and then washed three times with Hanks' solution before use.

Incubations—In the first series of experiments, in each experiment islets were distributed sequentially in cups of 1-ml volume to a total of 50 islets per cup. The cups hung from rubber stoppers closing 20-ml vials, and each cup contained 0.1 ml of Krebs-Ringer bicarbonate. In one cup [1,4-14C]succinate and in another [2,3-14C]succinate were at 1 mM concentration. In a third cup [1,4-14C]succinate and in a fourth [2,3-14C]succinate were at 10 mM concentration. The fifth cup contained [1-14C]acetate and the sixth [2-14C]acetate, also at 1 mM. The seventh cup had [2-14C]glucose and the eighth [6-14C]glucose at 20 mM. In a second series, the contents of the cups were identical except that in the first six cups there was also unlabelled glucose at 20 mM and in the remaining two unlabelled acetate at 1 mM. Each cup had 1.1 pCi of 14C in the labeled compound added, except for only 0.8 pCi of [2,3-14C]succinate. In a third series, one cup contained [1-14C]acetate and the other [2-14C]acetate, at 1 mM. Two other cups were the same except that the acetate was at 10 mM, and the last two cups also had labeled acetate at 10 mM but in the presence of 20 mM unlabelled glucose. In a fourth series labeled acetate was incubated with unlabelled glucose and unlabelled acetate with labeled glucose, but the islets were fresh, i.e., not cultured. Incubates were also prepared with identical contents except no islets were added. Vials with their contents were gassed with 95%-5% CO2 for 2 min and then kept at 37 °C for 2 h to absorb into the NaOH the CO2 evolved on addition of the acid.

The cups were removed and 2 ml of 5% BaCl2 were added to each vial. The barium carbonate that precipitated was collected by filtering under suction the contents of the vials into a preweighed filter paper. The barium carbonate that collected on the paper was washed with CO2-free water, dried and weighed. The barium carbonates weighed between 96 and 110 mg, about the theoretical yield from 0.5 mmol of NaHCO3.

The barium carbonate, still on filter paper, was placed at the bottom of a wide-mouth bottle containing 5 ml of water and closed with a rubber stopper from which a scintillation vial containing 2 ml of Hyamine was suspended. After evacuating air from the bottle through the stopper, 2 ml of 1 N H2SO4 was injected through the stopper into the water. The bottle with its contents was kept at 37 °C for 2 h to allow the CO2 evolved from the barium carbonate to be absorbed into the Hyamine. Scintillation fluid was then added and 14C activity assayed in a scintillation counter.

Calculations—Yields of 14CO2 in the incubates in the absence of islets were only 0.002–0.003% of the added 14C for all the labeled compounds, except 0.02% for [1,4-14C]succinate. Yields of 14CO2 in disintegrations/min were calculated by subtracting the relatively small number of disintegrations/min in CO2 collected in the absence of islets from the disintegrations/min in their presence. At concentrations of 1, 10, and 20 mM the quantities incubated were 100, 1000, and 2000 mM, respectively. Yields of 14CO2 expressed in nanomoles to 14CO2/mmol islets/90 min of incubation were calculated by multiplying the quantities incubated by the yields of 14CO2 in disintegrations/min and dividing by the disintegrations/min incubated. From the ratio of the yields from [1,4-14C]succinate to [2,3-14C]succinate, [1-14C]acetate to [2-14C]acetate, and [2-14C]glucose to [6-14C]glucose the fraction of the pyruvate carboxylated and decarboxylated that was carboxylated was calculated using Equation 1 (4),

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\frac{2(A^{14}CO_2) - (Pyr^{14}CO_2)}{1 + (Pyr^{14}CO_2) - (A^{14}CO_2)}
\]

where A14CO2 is the ratio from labeled acetate or succinate, Pyr14CO2 is the ratio from labeled glucose, and F, assumed to be 0.8, the ratio of 14C from [6-14C]glucose, the equivalent of [3-14C]pyruvate, in carbon 2 to carbon 3 of oxaloacetate (1, 2).

Statistical Analyses—Ratios and yields are recorded as mean ± S.E. The significance of differences between the ratios was assessed using Student’s t test for unpaired observations.

RESULTS

Yields of 14CO2 and the ratios of those yields in the first two series of experiments are recorded in Table I. The ratio of the 14CO2 yield from [2-14C]glucose to that from [6-14C]glucose at a glucose concentration of 20 mM was the same in the absence and presence of 1 mM acetate (means of 1.55 and 1.58). The ratios of the yields at 1 mM acetate were also the same in the absence and presence of 20 mM glucose (means of 2.64 and 2.72) and significantly more, p < 0.05 and p < 0.001, respectively, than the glucose ratios (1.55 and 1.58). The ratios of yields from the succinates were also significantly more than those from the labeled gluoses (p < 0.001, except p < 0.05 for 2.35 versus 1.55). The ratio at 1 mM succinate was not different from the ratio at 1 mM acetate in the absence (2.35 versus 2.64) but was in the presence of glucose (3.46 versus 2.72, p < 0.05). The ratios for succinate at 1 and 10 mM succinate were more in the presence than absence of 20 mM glucose (3.46 versus 2.35, p < 0.01, and 4.47 versus 3.05, p < 0.025). At 10 mM succinate in the presence of glucose the ratio was significantly more than for acetate (4.47 compared with 2.72, p < 0.01). At 10 mM succinate 3–5 times as much succinate was oxidized to CO2 as at 1 mM succinate. The increase in the ratio of the yields from succinate on unlabeled glucose addition was due mostly to a decrease in the yield of 14CO2 from [2,3-14C]succinate.

The ratios of 1.55 for Pyr14CO2 and 2.64 for A14CO2 calculate to a pyruvate carboxylation contribution of 43%. Using 2.35 for A14CO2 the contribution is 39% and using 3.05 it is 48%. The ratios of 1.58 for Pyr14CO2 and 2.72 for A14CO2 give a contribution of 43%; with 3.46 it is 49%, and with 4.47 it is 54%.

In the series of experiments comparing the ratios of yields at 1 and 10 mM acetate, and 10 mM acetate in the presence of glucose, there were no significant differences among the ratios (Table II). The ratio at 1 mM acetate in that series, 2.62 ± 0.24, was the same as in the first series, 2.64 ± 0.33 (Table I). In contrast to succinate, increasing acetate’s concentration 10-fold resulted in less than a 2-fold increase in its oxidation to CO2. Furthermore, the addition of glucose had no effect on the yield of 14CO2. Incubations of fresh islets with 1 mM acetate and 20 mM glucose (Table III) gave similar ratios to those of cultured islets. The ratios of 2.45 and 1.65, significantly different at p < 0.01, calculate to a carboxylation contribution of 35%.

DISCUSSION

In introducing the 14CO2 ratio method for estimating pyruvate carboxylation activity, Kelleher and Bryan (4) proposed and used in incubations of mitochondria [1,4-14C]succinate and [2,3-14C]succinate rather than [1-14C]acetate and [2-14C]acetate. They noted that the advantage was that succinate, unlike acetate, is not volatile and therefore does not produce large blank values for 14CO2 as generally collected. MacDonald (1–3) used the succinates because of high blanks he experienced on collecting 14CO2 formed from the labeled acetates. We eliminated high blanks by collecting CO2 in NaOH and then precipitating it as BaCO3. Since sodium acetate and barium acetate are water-soluble, [14C]acetate absorbed in the NaOH was re-
moved when the BaCO$_3$ was collected. The conditions of islet preparation and incubation were similar to those used by MacDonald (2, 3). Islets were cultured by him in medium containing 1, 5, 8, and 20 mM glucose rather than just 11 mM glucose.

Succinate used in a trace quantity, so that metabolism is not altered, should in theory be able to replace acetate. That is evidenced in the present study by 1) similar ratios at 1 and 10 mM succinate and acetate, 2) the greater increase in succinate's oxidation to CO$_2$ with the increase in concentration, and 3) the increase with glucose addition of the ratios with labeled succinate but not acetate.

Succinate is converted to oxaloacetate in the citric acid cycle (Fig. 2), and in one turn of the cycle succinate is regenerated, albeit containing the carbons of the acetyl-CoA that condensed with the oxaloacetate. Therefore, when succinate enters the cycle in substrate amounts, at steady state, the amount of carbon leaving the segment, equal to the amount in the succinate taken up, can only be as malate and/or aspartate (oxaloacetate and fumarate do not cross the inner mitochondrial membrane).

Following the transport of malate from the mitochondrial matrix to the cytosol, [1,4-$^{14}$C]malate would yield [1-$^{14}$C]pyruvate and $^{14}$CO$_2$, catalyzed by malic enzyme. [2,3-$^{14}$C]Malate would yield [2,3-$^{14}$C]pyruvate and no $^{14}$CO$_2$. Thus, the greater ratio of $^{14}$CO$_2$ yields, coupled with the 3–5-fold greater oxidation of the succinate to CO$_2$ at 10 mM than at 1 mM, with no source of exogenous acetyl-CoA, is evidence for much of the succinate at 10 mM being metabolized via the malic enzyme-catalyzed pathway. The increased ratio on glucose addition can be explained by dilution of labeled pyruvate by unlabeled pyruvate formed from the glucose. As a result, a smaller amount of labeled pyruvate formed from labeled succinate would be expected to be oxidized. The greater reduction in the yield of $^{14}$CO$_2$ from [2,3-$^{14}$C]succinate than from [1,4-$^{14}$C]succinate (Table I) is in accord with expectations. Indeed, if none of the labeled pyruvate was oxidized, $^{14}$CO$_2$ would be formed via the malic enzyme-catalyzed reaction from [1,4-$^{14}$C]succinate but not [2,3-$^{14}$C]succinate. In two of three incubations of 10 mM labeled succinate in the presence of unlabeled glucose, MacDonald (2) detected $^{14}$CO$_2$ formation from [1,4-$^{14}$C]succi-
nate but not \([2,3-^{14}C]\)succinate.

MacDonald demonstrated the presence of malic enzyme in islets (1, 5), Malaisse et al. (6), incubating islets with glutamine at a substrate concentration, 10 mM, reported that a major fraction of the glutamine, via conversion to \(\alpha\)-ketoglutarate, left the cycle as malate, which was converted to pyruvate. However, Malaisse and Sener (7) found similar ratios of yields of \([^{14}C]CO_2\), about 2, on incubating fresh islets with 2.8–16.7 mM \([2-^{14}C]\)glucose and \([6-^{14}C]\)glucose and with 1 mM \([1-^{14}C]\)acetate and \([2-^{14}C]\)acetate. This was the reason we incubated fresh islets by the paired flask technique.

The estimate using the paired glucose and acetate ratios, that about 40% of pyruvate entering the citric acid cycle was via carboxylation, is still near to the estimate of 54% substituting the 10 mM succinate ratios. Thus, the estimates are relatively insensitive to changes within the range of the \([^{14}C]CO_2\) ratios. \([2,4^{14}C]\)Glucose and \([6,4^{14}C]\)glucose are used, rather than \([2,14^{14}C]\)pyruvate and \([3,14^{14}C]\)pyruvate because of pyruvate's instability. Randomization of carbon 2 of glucose-6-P in the pentose cycle is assumed not to affect the ratio of the \([^{14}C]CO_2\) yields. The relative small contribution of the pentose cycle to glucose utilization by islets supports that assumption (2, 7). If there is significant conversion of dihydroxyacetone-3-P to glycerol or its derivatives, isotopic equilibration of the dihydroxyacetone-3-P with glyceraldehyde-3-P is assumed sufficiently complete so as not to result in an overestimation of carboxylation. Relative high activity of triose-P isomerase in islets (8), a relatively small incorporation of glucose carbon into lipid via glycerol 3-phosphate (9), and only slightly less yields of \([^{14}C]CO_2\) from \([6-^{14}C]\)glucose than \([1-^{14}C]\)glucose (2) support that assumption. Equal yields of \(3H_2O\) from \([2-^{3}H]\)glucose and \([5-^{3}H]\)glucose are not evidence for equilibration of the triose phosphates (2), since \(3H_2O\) is formed in the conversion of \([2-^{3}H]\)glucose-6-P to fructose-6-P and not in the equilibration, while \(3H\) from \([5-^{3}H]\)glucose not lost to \(3H_2O\) in the equilibration will be lost in the conversion of 2-phosphoglycerate to phosphoenolpyruvate.

The assumption that \(F = 0.8\) in the calculations, i.e. that there is extensive equilibration between oxaloacetate and fumarate, is supported by high activities of malic dehydrogenase and fumarase in islet mitochondria (2) and incorporations of about 80% as much \(^{14}C\) from \([3,4^{14}C]\)lactate into carbons 2 and 5 as carbons 1 and 6 of glucose formed by liver and kidney (10, 11). However, in the presence of a pool of unlabeled succinate formed from dimethyl succinate and a pool of labeled oxaloacetate formed from labeled pyruvate, isotopic equilibration of the dicarboxylic acids should be less than in the absence of the unlabeled succinate pool. An estimate for example of 54%, assuming \(F = 0.8\), decreases to 32% if there is no equilibration, \(F = 0\). An assumption of course is also that there are single pools of intermediates, e.g. acetate enters the same pool of acetyl-CoA as acetyl-CoA formed from pyruvate. That goes beyond any concern that the islet contains several cell types, even though β cells predominate.

In conclusion, the quantitation of pyruvate carboxylation in islets has been examined. It is estimated to proceed at about two-thirds the rate of pyruvate decarboxylation. The malate enzyme-catalyzed reaction allows for the removal from the citric acid cycle of the oxaloacetate formed. That is in accord with the recent report that when mitochondria from islets were incubated with \([1-^{14}C]\)pyruvate, \([^{14}C]\) was recovered in the incubation medium mainly in malate, and when islets were incubated with \([U-^{14}C]\)succinate \([^{14}C]\) appeared in pyruvate and lactate (12). Cytosolic NADPH would then be generated for cellular needs. There would then be cycling as the oxaloacetate, formed by fixation of pyruvate by \(CO_2\), is decarboxylated via malate and pyruvate is reformed (Fig. 3). A portion of the pyruvate would then be re-carboxylated to oxaloacetate with the remainder decarboxylated to acetyl-CoA and \(CO_2\) or reduced to lactate.

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