

[5-³H]glucose overestimates glycolytic flux in isolated working rat heart: role of the pentose phosphate pathway

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Goodwin, Gary W., David M. Cohen, and Heinrich Taegtmeier. [5-³H]glucose overestimates glycolytic flux in isolated working rat heart: role of the pentose phosphate pathway. *Am J Physiol Endocrinol Metab* 280: E502–E508, 2001.—We set out to study the pentose phosphate pathway (PPP) in isolated rat hearts perfused with [5-³H]glucose and [1-¹⁴C]glucose or [6-¹⁴C]glucose (crossover study with 1- then 6- or 6- then 1-¹⁴C-labeled glucose). To model a physiological state, hearts were perfused under working conditions with Krebs-Henseleit buffer containing 5 mM glucose, 40 μU/ml insulin, 0.5 mM lactate, 0.05 mM pyruvate, and 0.4 mM oleate/3% albumin. The steady-state C1/C6 ratio (i.e., the ratio from [1-¹⁴C]glucose to [6-¹⁴C]glucose) of metabolites released by the heart, an index of oxidative PPP, was not different from 1 (1.06 ± 0.19 for ¹⁴CO₂, and 1.00 ± 0.01 for [¹⁴C]lactate + [¹⁴C]pyruvate, mean ± SE, *n* = 8). Hearts exhibited contractile, metabolic, and ¹⁴C-isotopic steady state for glucose oxidation (¹⁴CO₂ production). Net glycolytic flux (net release of lactate + pyruvate) and efflux of [¹⁴C]lactate + [¹⁴C]pyruvate were the same and also exhibited steady state. In contrast, flux based on ³H₂O production from [5-³H]glucose increased progressively, reaching 260% of the other measures of glycolysis after 30 min. The ³H/¹⁴C ratio of glycogen (relative to extracellular glucose) and sugar phosphates (representing the glycogen precursor pool of hexose phosphates) was not different from each other and was <1 (0.36 ± 0.01 and 0.43 ± 0.05 respectively, *n* = 8, *P* < 0.05 vs. 1). We conclude that both transaldolase and the L-type PPP permit hexose detritiation in the absence of net glycolytic flux by allowing interconversion of glycolytic hexose and triose phosphates. Thus apparent glycolytic flux obtained by ³H₂O production from [5-³H]glucose overestimates the true glycolytic flux in rat heart.

transaldolase; glycogen; tracer kinetics

WE SET OUT TO STUDY the pentose phosphate pathway (PPP) in isolated working rat hearts and examined the hypothesis that ³H₂O release from [5-³H]glucose overestimates rates of glycolysis. The conventional view is that ³H₂O production can be used to measure glycolytic flux from [5-³H]glucose with loss of ³H into water at the triose phosphate isomerase reaction. Should this reaction be incomplete, the remainder would be lost at the enolase reaction (22). This line of reasoning assumes

that ³H₂O release from the nonoxidative portion of the pentose phosphate pathway can be disregarded.

The PPP functions mainly to synthesize ribose 5-phosphate (ribose 5-*P*) and NADPH. Ribose is used for synthesis of nucleic acids and nucleotide cofactors. The capacity for endogenous ribose synthesis by the heart is probably small, because provision of exogenous ribose is an intervention that stimulates resynthesis of the adenine nucleotide pool following conditions (e.g., ischemia) that deplete the pool (25, 33). Ribose may also be recycled. Compared with ribose, heart has a larger requirement for NADPH to counteract oxidative stress resulting especially from mitochondrial production of reactive oxygen species. The major requirement for NADPH in heart is to maintain reduced glutathione. Based on the distribution of enzyme activities, NADP⁺-dependent isocitrate dehydrogenase is a major source for NADPH synthesis in normal heart (1). In hypertrophied heart, the regulatory enzymes of the oxidative PPP [glucose 6-phosphate (G-6-*P*) dehydrogenase and 6-phosphogluconate dehydrogenase] are up-regulated and provide a further source for NADPH (34).

The PPP consists of two branches: an irreversible oxidative branch that produces NADPH and ribulose 5-*P*, and a reversible, nonoxidative branch that permits the interconversion of glycolytic intermediates with pentose phosphates, notably, ribulose 5-*P*, ribose 5-*P*, and xylulose 5-*P*. This last compound is of recent interest as a signaling molecule for transcriptional activation through the glucose response element (6) and stimulation of a type 2A protein phosphatase (23).

Horecker and Mehler (12) first noted the cyclical nature of the pathway created when G-6-*P* is oxidized to ribulose 5-*P* and then converted back to glycolytic intermediates (triose and hexose phosphates) by nonoxidative PPP (“pentose phosphate cycle” or “PPP with carbon recycling”). The nonoxidative branch of the PPP is required for synthesis of ribose 5-*P* from either ribulose 5-*P* or, in the absence of oxidative PPP, from glycolytic intermediates. Therefore, in the absence of an exogenous source of ribose, the heart has an absolute, albeit modest, requirement for the nonoxidative

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PPP. As a whole, the nonoxidative PPP adjusts the immediate requirement for ribose synthesis to that for NADPH.

In the present study, we used an existing method to assess the oxidative portion of the pathway on the basis of selective decarboxylation of glucose C1 by 6-phosphogluconate dehydrogenase (the C1/C6 ratio method, which is the relative rate of product formation from C1 ÷ C6 of glucose). Unlike previous studies, we measured the C1/C6 ratio for both $^{14}\text{CO}_2$ and released [^{14}C]lactate + [^{14}C]pyruvate, because $^{14}\text{CO}_2$ production alone is insufficient for rigorous interpretation of the oxidative pathway in the presence of carbon recycling. In addition, we provide new qualitative evidence regarding the operation of the nonoxidative pathway in the heart. The evidence for nonoxidative PPP stems from observations that we interpret as nonglycolytic detritiation of [$5\text{-}^3\text{H}$]glucose, reduced $^3\text{H}/^{14}\text{C}$ ratio of glycogen (and intracellular sugar phosphates, representing the glycogen precursor pool of hexose phosphates), and $^3\text{H}_2\text{O}$ production from [$5\text{-}^3\text{H}$]glucose (a measure of unidirectional glycolytic flux) in excess of glycolytic flux measured by other methods (net release of lactate + pyruvate, and efflux of [^{14}C]lactate + [^{14}C]pyruvate).

MATERIALS AND METHODS

Sources of materials. D-[$5\text{-}^3\text{H}$]glucose (product TRK.290, batch 42) was from Amersham (Arlington Heights, IL). The manufacturer's stated radiochemical and positional isotopic purities were 97% and "essentially 100%" (by tritium NMR), respectively. Owing to radiolysis, the product accumulates $^3\text{H}_2\text{O}$ on storage (~5%) at the time of the heart perfusions. For this reason, the purity of the compound was ascertained by column chromatography before onset of each experiment, and a small correction was applied to the calculated specific activity. D-[$1\text{-}^{14}\text{C}$]glucose (product G5645, lot 068H9446, $\geq 98\%$ purity) and D-[$6\text{-}^{14}\text{C}$]glucose (product G9899, lot 019H9616, $\geq 98\%$ purity) were from Sigma (St. Louis, MO). Sources of other materials were given previously (9).

Heart perfusions. Hearts from chow-fed male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN; 361 ± 2 g, $n = 8$) were perfused using a working heart apparatus (28). After pentobarbital sodium anesthesia (100 mg/kg ip) and heparin injection (100 U iv), hearts were removed and arrested in ice-cold perfusate. The aorta was cannulated, and retrograde perfusion was begun to wash out blood and cannulate the left atrium (~5 min). We then switched to a working mode (anterograde aortic flow) using recirculated perfusate (200 ml) for 5 min to obtain a stable preparation. The perfusion pressure (aortic afterload) was set at 73.5 mmHg (100 cmH $_2\text{O}$), and the atrial filling pressure in the working mode was 11.0 mmHg (15 cmH $_2\text{O}$). After 5 min, hearts were switched to a nonrecirculating mode supplied by fresh perfusate (600 ml for each 30-min interval) containing the indicated isotopes. The entire coronary flow was collected directly from the pulmonary artery into pre-weighed vials over 5-min intervals, and the vials were capped immediately. After 2 min to exchange the extracellular space, we began recirculating the aortic flow by redirecting the aortic overflow to the new reservoir. The aortic circuit was sampled at 10-min intervals to measure blank values, and the content of metabolites calculated for the coronary flow was corrected for average blanks measured in the recirculated aortic circuit. The perfusate consisted of warm (37°C)

Krebs-Henseleit buffer with 5 mM glucose, 40 $\mu\text{U/ml}$ regular insulin (Humulin, Eli Lilly, Indianapolis, IN), 0.5 mM sodium L-lactate, 0.05 mM sodium pyruvate, 1.5 mM CaCl $_2$, 0.1 mM EDTA (~1.4 mM free Ca $^{2+}$) and was equilibrated with 95% O $_2$ -5% CO $_2$. The perfusate during the nonrecirculating perfusion also contained 0.4 mM sodium oleate prebound to 3% bovine serum albumin (fatty acid free, Intergen, Purchase, NY), 1.4 mM free Ca $^{2+}$, 0.025 $\mu\text{Ci/ml}$ [$5\text{-}^3\text{H}$]glucose, and either 0.01 $\mu\text{Ci/ml}$ of [$1\text{-}^{14}\text{C}$]glucose or 0.01 $\mu\text{Ci/ml}$ of [$6\text{-}^{14}\text{C}$]glucose. The free calcium was adjusted by overnight dialysis against 10 volumes of albumin-free perfusate containing 1.5 mM CaCl $_2$ and 0.1 mM EDTA. Perfusions were continued for 30 min in the presence of one of the two [^{14}C]glucose isotopomers and then switched to a second reservoir containing the alternate ^{14}C isotopomer, and the perfusions were continued for another 30 min before free-clamping the hearts on the cannula with aluminum tongs cooled in liquid N $_2$. There were eight perfusions total (4 for each order of addition of [$1\text{-}^{14}\text{C}$] or [$6\text{-}^{14}\text{C}$]glucose). [$5\text{-}^3\text{H}$]glucose was present at constant specific activity throughout.

Analytical procedures. All metabolites measured in the coronary flow are duplicates. $^3\text{H}_2\text{O}$ was separated from [$5\text{-}^3\text{H}$]glucose in samples of fresh perfusate (0.5 ml) by use of 1.5-ml columns of AG 1-X8 resin (hydroxide form) as described previously (8). The assay for [^{14}C]lactate plus [^{14}C]pyruvate in samples (0.3 ml) of deproteinized perfusate, with the use of paper chromatography to separate [^{14}C]glucose, was described previously (8). The recovery of [^{14}C]lactate for the entire procedure, by means of fresh perfusate spiked with authentic [$\text{U}\text{-}^{14}\text{C}$]lactate, is 80%, and lactate is completely separated from [$\text{U}\text{-}^{14}\text{C}$]glucose (8). Total lactate and pyruvate were measured enzymatically in deproteinized perfusate by means of standard enzymatic assays with lactate dehydrogenase (2). To measure $^{14}\text{CO}_2$, 10-ml portions of fresh perfusate were transferred to 50-ml Erlenmeyer flasks, and the flasks were fitted with a serum cap and hanging center well (Kontes, Vineland, NJ) containing a strip of fluted filter paper and 0.5 ml of $^{14}\text{CO}_2$ -trapping agent (1 M hyamine hydroxide in methanol). The perfusate was acidified by injecting 0.5 ml of 60% perchloric acid (PCA). The vials were incubated overnight, and the wells were measured for radioactivity in 10 ml of scintillation mixture (Ultima Gold, Packard, Meriden, CT). Metabolic rates were calculated from the content of radioactivity in the coronary flow (dpm/ml, corrected for blanks determined on the recirculated aortic circuit) divided by the specific activity measured in the aortic circuit (dpm/ μmol), multiplied by the coronary flow rate (ml/min), and normalized to the dry weight of each heart. Fluxes for total lactate and pyruvate, measured enzymatically, were calculated from the concentration difference across the heart times the coronary flow and are expressed as glycosyl units (one-half the lactate + pyruvate flux). Flow rates were calculated from the filling time for an in-line graduated chamber (aortic flow) or gravimetrically (coronary flow). Contractile performance is expressed as the rate of external pressure-volume work (hydraulic power). Average power (watts) is the product of cardiac output (aortic plus coronary flow, m 3 /s) multiplied by the pressure differential (afterload - preload) across the heart in pascal units (73.5 - 11.0 = 62.5 mmHg, 8,500 Pa). In this preparation, the mean aortic pressure (calculated as one-third systolic + two-thirds diastolic pressure) assumes a value equal to the afterload, which is fixed by the height of the aortic overflow above the aortic valve (100 cm). Other measures of contractile performance (Table 1) were measured with a 3 French Millar pressure transducer (Millar Instruments, Houston, TX) at the side arm of the aortic cannula interfaced to a Gould

Table 1. *Contractile activity of the two isotope treatment groups*

Parameter	Isotope Treatment Group	
	[1- ¹⁴ C]Glucose	[6- ¹⁴ C]Glucose
Heart rate, min	248 ± 21	255 ± 21
Coronary flow, ml/min	16.9 ± 0.8	17.6 ± 2.2
Aortic flow, ml/min	27.4 ± 2.2	28.5 ± 2.5
Cardiac output, ml/min	44.1 ± 2.1	45.6 ± 2.2
Hydraulic power, mW	6.13 ± 0.29	6.33 ± 0.31
Peak aortic pressure, mmHg	106.8 ± 6.1	106.4 ± 5.3
Developed aortic pressure, mmHg	50.3 ± 6.2	49.8 ± 5.7
Rate × peak pressure, mmHg/min	25,900 ± 1300	26,600 ± 1100
Rate × developed pressure, mmHg/min	11,900 ± 1000	12,100 ± 700

Values are means ± SE ($n = 8$ observations with each ¹⁴C isotope of glucose). Values for each perfusion are the means of measurements taken at 5-min intervals.

physiological recorder (Gould model 2400S; Cleveland, OH). Hearts were weighed and ground to a fine powder under liquid nitrogen, and a portion of the powder was used to measure dry weight. Glycogen was isolated from the powdered tissue by means of a standard procedure (29). Isolated glycogen was dissolved in acetate buffer (0.5 ml), digested to glucose with amyloglucosidase (to avoid a ³H-quenching artifact) following a standard procedure (2), and a portion was mixed with 10 ml of scintillation mixture for dual isotope scintillation counting. For total glycogen (as glucosyl units), the digest was assayed for glucose by use of hexokinase and glucose-6-phosphate dehydrogenase (2). In one experiment, a sample of isolated glycogen from 5 mg dry wt of heart containing 400 dpm tritium and 609 dpm ¹⁴C was added to 50 mg wet wt of powdered heart (nonradioactive), and the glycogen isolation procedure was repeated before the sample was subjected to digestion with amyloglucosidase. The reisolated glycogen contained 369 dpm tritium (93% recovery) and 518 dpm ¹⁴C (83% recovery). The result indicated that selective detritiation during glycogen isolation was negligible. Intracellular sugar phosphates were isolated from fresh 6% PCA extracts of powdered heart (200 mg) following a published procedure (10). The neutralized PCA extracts were added to 0.25 g of Dowex 2 × 8–100 (50–100 mesh) suspended in 4 ml of 0.17 M NH₄OH, incubated for 1 h, and centrifuged, and the supernatant was aspirated. The resin was washed four times with 4 ml of 0.17 M NH₄OH and then treated with 1 ml of 1 M HCl. A 0.5-ml aliquot of the acid eluent containing hexose phosphates and other phosphorylated sugars was added to 10 ml of scintillation mixture to measure the ³H/¹⁴C ratio by dual isotope scintillation counting. Values were corrected for blanks measured in each heart by use of deproteinized extracts of coronary flow collected near the end of the heart perfusions. Another portion of PCA extract of the hearts was used for enzymatic assay of G-6-P with glucose-6-phosphate dehydrogenase (2). For radiochemical analysis, quench correction and simultaneous determination of ³H and ¹⁴C were performed by a routine (spectral index analysis) supplied with the instrument (Packard 1900 TR).

Data are expressed as means ± SE. Statistical comparison of rates from glucose C1 vs. C6 and incorporation of ³H vs. ¹⁴C were by Student's *t*-test for paired data. Glycolytic flux (Fig. 4) was compared by analysis of variance, with post hoc

comparison by the Newman-Keuls test. $P < 0.05$ was considered significant.

RESULTS

Contractile performance. We conducted two sets of four heart perfusions, each of 1 h total duration (30 min with one [¹⁴C]glucose isotopomer and then 30 min with the other). The perfusions were identical in every respect except for the order of addition of the two ¹⁴C isotopomers of glucose. We included [1-¹⁴C]glucose first and then [6-¹⁴C]glucose, or vice versa (crossover study design). [5-³H]glucose was included throughout both sets. Contractile performance, measured as hydraulic power (the product of cardiac output and the mean pressure differential across the heart) throughout the protocol for both sets, is shown in Fig. 1. The two sets were well matched for performance, and all perfusions exhibited stable performance for the duration of the protocol. Table 1 gives additional measures of contractile activity: heart rate, coronary and aortic flow, cardiac output, hydraulic power, maximum and minimum aortic pressure, developed pressure, and rate × pressure products. Consistent with the crossover design of the study, the contractile data given in Table 1 are grouped according to which ¹⁴C isotopomer of glucose was present. Again, the two groups were well matched for performance (Table 1). This is necessary to obtain meaningful values for the C1/C6 ratios calculated pairwise (i.e., rates from C1 and C6 measured with the same heart).

Metabolic activity for [1-¹⁴C]- and [6-¹⁴C]glucose. Figure 2 shows rates of oxidation (¹⁴CO₂ production), and Fig. 3 shows rates of release of [¹⁴C]lactate plus [¹⁴C]pyruvate during the time course of the perfusions with a given [¹⁴C]glucose isotopomer. In each figure, values from [1-¹⁴C]glucose and from [6-¹⁴C]glucose are shown side by side. Consistent with the crossover design of the study, data were grouped according to which ¹⁴C isotopomer of glucose was present, as in Table 1.

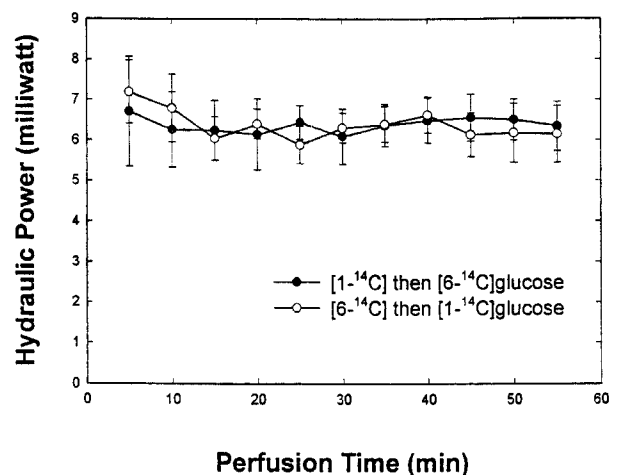


Fig. 1. Contractile performance of the two groups of 60-min heart perfusions. One group was with [1-¹⁴C]glucose for the first 30 min and then [6-¹⁴C]glucose for the second 30 min and vice versa for the other group. Values are means ± SE ($n = 4$ perfusions each).

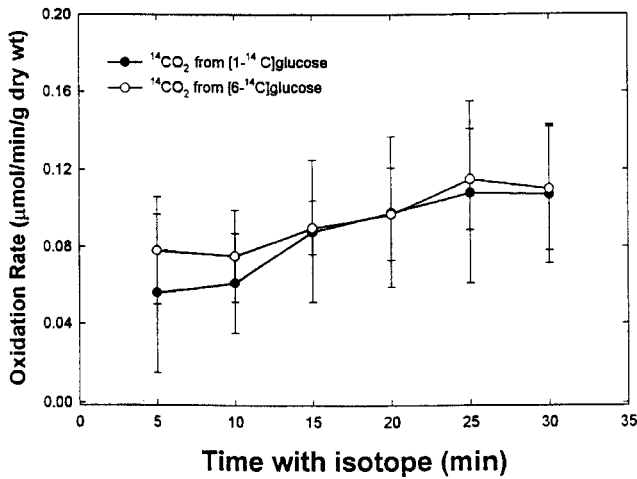


Fig. 2. Rates of $^{14}\text{CO}_2$ production (oxidation) during time of perfusion with a given ^{14}C isotopomer of glucose. (●), Oxidation of [1- ^{14}C]glucose. (○), Oxidation of [6- ^{14}C]glucose. Values are means \pm SE ($n = 8$ observations with each ^{14}C isotopomer of glucose).

Values were calculated on the basis of the specific activity of extracellular glucose and are reported in terms of glycosyl (C6) units. In both cases (oxidation and ^{14}C glycolytic flux), hearts reached isotopic/metabolic steady state within the time frame of perfusion with a given ^{14}C isotopomer (30 min), allowing us to calculate steady-state values for the C1/C6 ratios. Both ratios were calculated from the average rate for the last three time points (Fig. 2). The steady-state C1/C6 ratio was 1.06 ± 0.19 ($n = 8$) for $^{14}\text{CO}_2$ and 1.00 ± 0.01 ($n = 8$) for [^{14}C]lactate + [^{14}C]pyruvate. In other words, rates of glucose oxidation and unidirectional glycolytic ^{14}C carbon flux from glucose C1 and from C6 were indistinguishable.

A characteristic feature of the isolated working heart (but not necessarily of the heart in vivo) is very high glycolytic activity with pronounced release of lactate, and to a lesser extent pyruvate, into the coronary flow

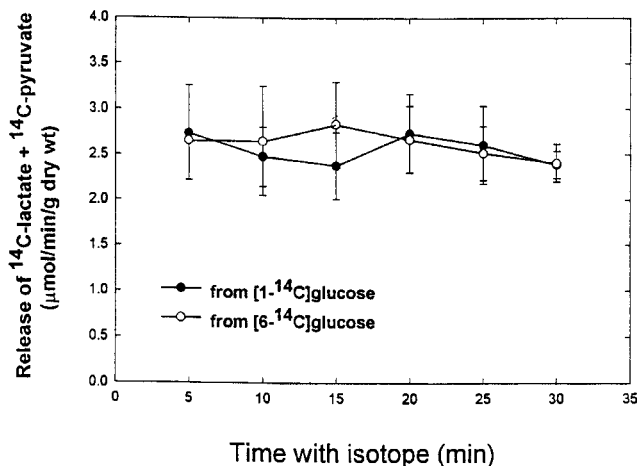


Fig. 3. Rates of efflux of [^{14}C]lactate + [^{14}C]pyruvate during time of perfusion with a given ^{14}C isotopomer of glucose. Values are in glycosyl (C6) units. (●), Perfusion with [1- ^{14}C]glucose. (○), Perfusion with [6- ^{14}C]glucose. Values are means \pm SE ($n = 8$ observations with each ^{14}C isotopomer of glucose).

(Figs. 3 and 4). At the same time, exogenous lactate and pyruvate are good respiratory substrates for the heart. For this reason, our heart perfusions were performed without recirculation of the coronary flow. If the coronary flow were allowed to reenter a recirculated circuit, hearts could not be expected to achieve isotopic or metabolic steady state, because the accumulation of lactate and pyruvate in a recirculated system would change their concentration and specific activity.

Glycolytic flux. Figure 4 shows three measures of glycolytic flux during the time course of perfusion. One of the lower two plots shows net release of lactate + pyruvate (in C6 units) based on concentration differences across the heart, measured enzymatically. The separate contributions by lactate and pyruvate to net flux averaged 2.2 and 0.4 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}$ dry wt $^{-1}$ (C6 units), respectively. The second of the two lower plots shows efflux of [^{14}C]lactate + [^{14}C]pyruvate from [^{14}C]glucose (the average of the two rates given in Fig. 3). We did not report separate values for efflux of [^{14}C]lactate and [^{14}C]pyruvate because the radiochemical assay does not separate lactate from pyruvate. Strictly speaking, efflux of [^{14}C]lactate + [^{14}C]pyruvate is a unidirectional process that may differ from net flux of lactate + pyruvate measured enzymatically because of exchange between intracellular (metabolically derived) lactate and extracellular lactate (7, 8). The exchange contributes to ^{14}C efflux but not to net flux. However, net flux and efflux did not differ in the present study (Fig. 4). The third measure of glycolytic flux shown in Fig. 4 is $^3\text{H}_2\text{O}$ production from [5- ^3H]glucose. Like efflux of [^{14}C]lactate + [^{14}C]pyruvate, $^3\text{H}_2\text{O}$ production measures a unidirectional process. Because the 5-position is first detritiated at the level of a triose (specifically, at triose phosphate isomerase, since this is an equilibrium enzyme), $^3\text{H}_2\text{O}$ production from [5- ^3H]glucose should measure glycolytic flux from glucose to trioses, at least in the absence of confounding processes like gluconeogenesis or

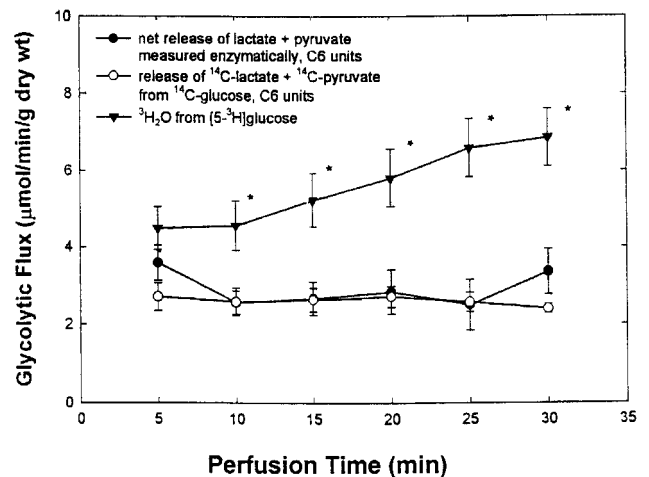


Fig. 4. Glycolytic flux measured by different methods during the time course of perfusion. All values are in glycosyl (C6) units. (●), Net flux of lactate + pyruvate in glycosyl (C6) units, measured enzymatically; (○), efflux of [^{14}C]lactate + [^{14}C]pyruvate from [1- ^{14}C] and [6- ^{14}C]glucose. Values are the average of the rates shown in Fig. 3. (▼), Glycolytic flux based on $^3\text{H}_2\text{O}$ production from [5- ^3H]glucose. Values are means \pm SE ($n = 8$). * $P < 0.05$ compared with the other groups.

transaldolase. The rate should include both glucose oxidation and glycolytic flux appearing as lactate + pyruvate in the coronary flow, although the contribution by glucose oxidation ($0.1 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g dry wt}^{-1}$, Fig. 2) was negligible compared with total flux ($2.6 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g dry wt}^{-1}$). Therefore, we would expect the rate for $^3\text{H}_2\text{O}$ production to equal the other measures of glycolytic flux shown in Fig. 4 plus a very small contribution by glucose oxidation, shown in Fig. 2, in the absence of confounding reactions. Contrary to this expectation, the rate of $^3\text{H}_2\text{O}$ production did not exhibit steady state. The rate increased progressively during the protocol and reached 260% of the other measures of glycolytic flux after 30 min (Fig. 4). We interpret this result as evidence for detritiation of $[5\text{-}^3\text{H}]\text{glucose}$ in excess of true glycolytic flux because of the presence of confounding reactions.

Tritium and ^{14}C incorporation into intracellular metabolites. Table 2 shows tritium and ^{14}C radioactivity recovered in glycogen (*top*) and cellular phosphorylated sugars (*bottom*), as well as the content of glycogen and G-6-P in the two groups of heart perfusions. The *left* column shows hearts perfused with $[1\text{-}^{14}\text{C}]$ - and then $[6\text{-}^{14}\text{C}]\text{glucose}$, and the *right* column shows hearts perfused with $[6\text{-}^{14}\text{C}]$ - and then $[1\text{-}^{14}\text{C}]\text{glucose}$. $[1\text{-}^{14}\text{C}]$ - and $[6\text{-}^{14}\text{C}]\text{glucose}$ were included at the same specific activity, and $[5\text{-}^3\text{H}]\text{glucose}$ was present at constant specific activity throughout the protocol. Using values for ^3H and ^{14}C specific activity of extracellular glucose given in the legend to Table 2, we calculated the relative enrichment of glycogen and cellular sugar phosphates for the $[5\text{-}^3\text{H}]\text{glycosyl}$ moiety compared with the $[^{14}\text{C}]\text{glycosyl}$ moiety. We expected $[^{14}\text{C}]$ - and $[^3\text{H}]\text{glycosyl}$ enrichments of glycogen to be the same. Contrary to the expectation, the amount of new glycogen synthesis based on incorporation of tritium was, on average, 36% of the value based on incorporation of ^{14}C ($[^3\text{H}]/[^{14}\text{C}]\text{glycosyl}$ enrichment ratio of 0.33 ± 0.02 and 0.38 ± 0.01 in Table 2, $P < 0.05$ vs. 1). The result indicates

that one or more intermediates between glucose and glycogen were detritiated.

The $^3\text{H}/^{14}\text{C}$ glycosyl enrichment ratio of cellular sugar phosphates was measured as an indication of the enrichment ratio for the glycogen precursor pool of hexose phosphates. The value should be interpreted with caution, however, because we did not isolate hexose phosphates, and only a portion of cellular sugar phosphates was accounted for as G-6-P (*bottom* half of Table 2). The enrichment ratios for cellular sugar phosphates given in Table 2 (0.40 ± 0.05 and 0.45 ± 0.05 , $P < 0.05$ vs. 1) were not different from the enrichment ratio for glycogen and were also < 1 . Glycogen is synthesized from a pool of hexose phosphates (G-1-P, G-6-P and fructose 6-P), which are at least partially equilibrated, isotopically, since phosphoglucomutase and phosphoglucose isomerase are reversible enzymes. G-6-P and fructose 6-P are probably close to equilibrated with each other (17). None of the enzymatic reactions of glycogen synthesis from hexose phosphates or reactions of glycogen remodeling cause detritiation of the glycosyl 5-position, to our knowledge. Therefore, the finding that glycogen and the hexose phosphate pool exhibit the same $^3\text{H}/^{14}\text{C}$ enrichment ratio is not an unexpected result. Our explanation for the observation that the $^3\text{H}/^{14}\text{C}$ ratio of both glycogen and the glycogen precursor pool are < 1 is that one or more hexose phosphates were detritiated before incorporation into glycogen, which is consistent with the observations (see *Glycolytic flux*) of overestimated glycolytic flux based on $^3\text{H}_2\text{O}$ production from $[5\text{-}^3\text{H}]\text{glucose}$.

DISCUSSION

The main finding of this study is a discrepancy between the apparent rate of glycolysis as determined by a popular isotopic tracer method and the true rate of glycolysis as determined by a second, independent

Table 2. Radiochemical enrichment of glycogen and intracellular sugar phosphates from $[5\text{-}^3\text{H}]\text{glucose}$ and $[^{14}\text{C}]\text{glucose}$

	Isotopes Present During Experiment ($[5\text{-}^3\text{H}]\text{glucose}$ throughout)	
	$[1\text{-}^{14}\text{C}]$ - then $[6\text{-}^{14}\text{C}]\text{glucose}$	$[6\text{-}^{14}\text{C}]$ - then $[1\text{-}^{14}\text{C}]\text{glucose}$
<i>Glycogen</i>		
Glycogen content, $\mu\text{mol/g dry wt}$	82 ± 19	92 ± 3
Tritium counts in glycogen, $\text{dpm/g dry wt} \times 10^{-3}$	80.4 ± 2.3	71.9 ± 2.4
^3H glycosyl enrichment of glycogen, $\mu\text{mol/g dry wt}$	12.1 ± 0.3	10.4 ± 0.3
^{14}C counts in glycogen, $\text{dpm/g dry wt} \times 10^{-3}$	120 ± 12	88.2 ± 1.5
$[^{14}\text{C}]\text{glycosyl}$ enrichment of glycogen, $\mu\text{mol/g dry wt}$	36.5 ± 2.0	27.3 ± 0.2
$[^3\text{H}]\text{glycosyl}$ enrichment \div $[^{14}\text{C}]\text{glycosyl}$ enrichment	$0.33 \pm 0.02^*$	$0.38 \pm 0.01^*$
<i>Sugar phosphates</i>		
G-6-P, $\mu\text{mol/g dry wt}$	1.22 ± 0.24	1.37 ± 0.22
^3H counts in sugar phosphates, $\text{dpm/g dry wt} \times 10^{-3}$	19.6 ± 1.5	19.7 ± 1.6
$[^3\text{H}]\text{glycosyl}$ enrichment of sugar phosphates, $\mu\text{mol/g dry wt}$	2.80 ± 0.26	2.74 ± 0.27
^{14}C counts in sugar phosphates, $\text{dpm/g dry wt} \times 10^{-3}$	19.7 ± 1.6	21.3 ± 0.9
$[^{14}\text{C}]\text{glycosyl}$ enrichment of sugar phosphates, $\mu\text{mol/g dry wt}$	6.99 ± 0.10	6.20 ± 0.57
$[^3\text{H}]\text{glycosyl}$ enrichment \div $[^{14}\text{C}]\text{glycosyl}$ enrichment	$0.40 \pm 0.05^*$	$0.45 \pm 0.05^*$

Values are means \pm SE ($n = 4$ perfusions in each group). G-6-P, glucose 6-phosphate. *Different from 1 ($P < 0.05$ by paired t -test of $[^3\text{H}]/[^{14}\text{C}]\text{glycosyl}$ enrichment). Enrichments were calculated on the basis of the specific activity of extracellular glucose: 6,800 dpm/ μmol for $[5\text{-}^3\text{H}]\text{glucose}$, and 3,260 dpm/ μmol for both $[1\text{-}^{14}\text{C}]$ - and $[6\text{-}^{14}\text{C}]\text{glucose}$.

method. Under present experimental conditions, the rate of detritiation continued to increase over 20 min, whereas the rate of glycolysis estimated from the rate of ^{14}C release in lactate and pyruvate remained constant. We also observed a loss of $\sim 60\text{--}70\%$ of ^3H relative to ^{14}C in the glycosyl moieties of glycogen, suggesting a loss of tritium in the nonoxidative PPP.

These observations are the result of an exploration of the PPP in heart. Indeed, the nonoxidative PPP turned out to be a major source of $^3\text{H}_2\text{O}$ from $[5\text{-}^3\text{H}]\text{glucose}$. Futile cycling between hexose and triose phosphate is unlikely to account for the loss of tritium, because the activity of fructose-1,6-bisphosphatase is negligible in the heart (24). Therefore, the discussion will focus on the PPP.

Evidence for nonoxidative PPP. Our evidence for nonoxidative PPP is qualitative and is based on the following two observations. First, we found that $^3\text{H}_2\text{O}$ production from $[5\text{-}^3\text{H}]\text{glucose}$, unlike the other two measures of glycolytic flux, did not exhibit steady state and progressively overestimated the other measurements of glycolysis (net release of lactate + pyruvate and efflux of $[^{14}\text{C}]\text{lactate} + [^{14}\text{C}]\text{pyruvate}$ from $[^{14}\text{C}]\text{glucose}$). We interpret this result as detritiation in the absence of net glycolysis. Second, we found that the enrichment of glycogen (and the glycogen precursor pool of hexose phosphates, represented by cellular phosphorylated sugars) was considerably less when based on incorporation of tritium from $[5\text{-}^3\text{H}]\text{glucose}$ compared with incorporation of ^{14}C from $[1\text{-}^{14}\text{C}]$ - or $[6\text{-}^{14}\text{C}]\text{glucose}$. It was recognized early (13, 18) that this pattern of $^3\text{H}_2\text{O}$ production and tritium incorporation from $[5\text{-}^3\text{H}]\text{glucose}$ can occur in tissues like liver, which either exhibit the substrate cycle involving the enzyme fructose-1,6-bisphosphatase (which is undetectable in heart) and/or exhibit transaldolase activity, because both processes allow interconversion of glycolytic triose and hexose phosphates. The enzyme transaldolase (E.C. 2.2.1.2) detritiates C5 of fructose 6-phosphate. Transaldolase activity in rat heart has been reported to be 14–53% of the activity in liver (11, 14).

Because the glucose 5-position exchanges tritium with the solvent at the level of a triose, nonoxidative PPP-catalyzed isotopic exchange between glycolytic triose phosphates and hexose phosphates should be manifested as detritiation of glycolytic hexoses, which we measured in terms of the $^3\text{H}/^{14}\text{C}$ ratio of glycogen and intracellular glycogen precursors and in terms of $^3\text{H}_2\text{O}$ production in excess of what we believe to be true glycolytic flux.

At least two nonoxidative pathways have been proposed, F-type and L-type, for fat and liver, respectively (F-type is the classical, textbook presentation). Severim and Stepanova (27) and Williams (reviewed in Ref. 29) provided evidence for an alternate (L-type) pathway that does not require transaldolase. The evidence for this pathway is controversial (20, 32). The precise reaction sequence that constitutes the (putative) L-type pathway is not completely resolved and would vary among tissues [different pathways are required to accommodate the difference in specificity of liver vs. muscle aldolase, for example (3)]. On the basis of the pattern of

carbon redistribution from glucose C2 (or ribose C1) into hexose C1 and C3, it was concluded that the L-type, but not the F-type pathway, is active in the heart (15, 31). More recently, enzyme activities for both oxidative and nonoxidative pathways, including transaldolase, were found at moderate levels associated with heart sarcoplasmic reticulum (4), suggesting compartmentation of an active pathway.

Appraisal of oxidative PPP in heart based on C1/C6 ratios. Interpretation of the C1/C6 ratio is a complicated issue. During glycolysis, C1 and C6 of glucose become pyruvate C3. In contrast, some of glucose C1 can be decarboxylated before reaching pyruvate, at the reaction catalyzed by 6-phosphogluconate dehydrogenase. In the presence of oxidative PPP, the overall rate of $^{14}\text{CO}_2$ production from glucose C1 depends on the extent of carbon recycling. If there is extensive recycling of glucose-derived ribulose 5-*P* back into the glycolytic pathway (not unlikely for normal heart), then $^{14}\text{CO}_2$ production from glucose C1 and C6 will be the same ($^{14}\text{CO}_2$ C1/C6 = 1) irrespective of the activity of oxidative PPP. Therefore, it is not possible to draw a definitive conclusion regarding the oxidative PPP from $^{14}\text{CO}_2$ data alone. A different set of considerations applies to release of $[^{14}\text{C}]\text{lactate} + [^{14}\text{C}]\text{pyruvate}$. Oxidative PPP with carbon recycling decreases the specific activity of the glycolytic pathway from glucose C1 but not C6. Recycled carbon from $[1\text{-}^{14}\text{C}]\text{glucose}$ does not contribute to ^{14}C in lactate or pyruvate at all. Stated differently, decreased specific activity of the glycolytic pathway from glucose C1 relative to C6 because of carbon recycling should be detectable from the C1/C6 ratio of $[^{14}\text{C}]\text{lactate} + [^{14}\text{C}]\text{pyruvate}$ but not necessarily from $^{14}\text{CO}_2$, because the decrease in glycolytic specific activity and the action of 6-phosphogluconate dehydrogenase oppose each other with respect to the overall rate of $^{14}\text{CO}_2$ production from $[1\text{-}^{14}\text{C}]\text{glucose}$. For this reason, we included measurements, lacking in previous studies of heart PPP, required to interpret the oxidative PPP rigorously based on C1/C6 ratios (i.e., we measured both $^{14}\text{CO}_2$ and $[^{14}\text{C}]\text{lactate} + [^{14}\text{C}]\text{pyruvate}$).

The C1/C6 ratio of $^{14}\text{CO}_2$ from heart was measured in two previous studies, with conflicting results. First, using isolated working hearts, Pfeiffer et al. (26) could not detect substrate flux through oxidative PPP. This study did not measure glucose oxidation directly; rather, it was based on a very indirect approach employing $^{14}\text{CO}_2$ washout kinetics. The second study used isolated heart myocytes (5). The addition of competing substrates (pyruvate, octanoate, etc.) to myocytes increased the C1/C6 ratio of $^{14}\text{CO}_2$ to a value >1 . From this result, the authors concluded that there is an appreciable contribution of oxidative PPP to total glucose oxidation under conditions where mitochondrial glucose oxidation is suppressed by the other substrates (the usual situation in vivo). The authors' interpretation was criticized in a subsequent editorial reply by Katz (16), who reiterated the points, from the original work of Katz and Wood (19) and a more recent reappraisal by Larabee (21), that the C1/C6 ratio of $^{14}\text{CO}_2$ is of limited quantitative value in the absence of addi-

tional measures of glucose utilization. We have provided the missing measurements in the present study. Larabee also noted potential problems associated with tissue metabolic heterogeneity, which probably apply to virtually any metabolic measurement in a heterogeneous system. This is not a moot issue in the case of the isolated working rat heart, because we previously found evidence for heterogeneity of carbohydrate metabolism for glucose and glycogen in this system (9). In any event, because we found that the C1/C6 ratio for both $^{14}\text{CO}_2$ and for [^{14}C]lactate + [^{14}C]pyruvate is equal to 1 (the rates from [1- ^{14}C]glucose and [6- ^{14}C]glucose were indistinguishable), we suggest that flux for oxidative PPP, although not necessarily absent, is small compared with overall glucose oxidation.

In summary, we have found $^3\text{H}_2\text{O}$ production from [5- ^3H]glucose in excess of glycolytic flux and decreased isotope incorporation into glycogen from [5- ^3H]glucose compared with [^{14}C]glucose. We interpret the results as evidence for nonoxidative PPP in heart, consistent with transaldolase activity previously reported in heart sarcoplasmic reticulum. The steady-state rate of production of $^{14}\text{CO}_2$ and of [^{14}C]lactate + [^{14}C]pyruvate was the same from [1- ^{14}C]glucose compared with [6- ^{14}C]glucose, suggesting that oxidative PPP is small compared with overall glucose oxidation.

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