Flux control in the rat gastrocnemius glycogen synthesis pathway by in vivo $^{13}$C/$^{31}$P NMR spectroscopy

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Flux control in the rat gastrocnemius glycogen synthesis pathway by in vivo $^{13}$C/$^{31}$P NMR spectroscopy. Am J Physiol Endocrinol Metab 280: E598–E607, 2001.—To determine the relative contributions of glucose transport/hexokinase, glycogen synthase (GSase), and glycolysis to the control of insulin-stimulated muscle glycogen synthesis, we combined $^{13}$C and $^{31}$P NMR to quantify the glycogen synthesis rate and glucose 6-phosphate (G-6-P) levels in rat (Sprague-Dawley) gastrocnemius muscle during hyperinsulinemia at euglycemic (E) and hyperglycemic (H) glucose concentrations under thiopental anesthesia. Flux control was calculated using metabolic control analysis. The combined control coefficient of glucose transport/hexokinase (GT/Hk) for glycogen synthesis was 1.1 ± 0.03 (direct measure) and 1.14–1.16 (calculated for a range of glycolytic fluxes), whereas the control coefficient for GSase was much lower (0.011–0.448). We also observed that the increase in in vivo [G-6-P] from E to H (0.22 ± 0.03 to 0.40 ± 0.03 mM) effects a supralinear increase in the in vitro velocity of GSase, from 14.6 to 26.1 mU·kg$^{-1}$·min$^{-1}$ (1.8-fold). All measurements suggest that the majority of the flux control of muscle glycogen synthesis is at the GT/Hk step.

metabolic control analysis; glucose transport; hyperglycemia; hyperinsulinemia

THE MUSCLE GLYCOGEN SYNTHESIS PATHWAY plays a central role in whole body glucose homeostasis. The flux through this pathway is acutely activated by insulin after a glucose load, allowing increased uptake of plasma glucose. Under the conditions of a hyperinsulinemic hyperglycemic clamp, muscle glycogen synthesis accounts for the majority of whole body glucose uptake in humans (29). Impaired glycogen synthesis accounts for the deficit of glucose storage in patients with non-insulin-dependent diabetes mellitus under these conditions (29). Impaired glycogen synthesis and the extensive regulation of GSase activity by phosphorylation have been the basis for the view of GSase as controlling the glycogen synthesis flux.

A limitation of previous studies of the muscle glycogen synthesis pathway is that they have not quantitatively assessed metabolic control. In the last 25 years, metabolic control analysis (MCA) has provided a theoretical framework for quantitatively calculating from experimental data the control exerted by enzymes over the flux through a metabolic pathway (12, 16). In MCA, the control an enzyme (E) exerts over a metabolic flux (J) is given by the flux control coefficient, $C_{E}^{J}$, which is defined as the fractional change in the flux through the pathway (12, 16).

$$C_{E}^{J} = (\delta J / J) / (\delta E / E)$$  \hspace{1cm} (1)$$

Measurement of the flux control coefficient of an enzyme depends on selectively altering the activity of the enzyme while maintaining constant activities of other enzymes of GT (14, 20, 37), GSase (7, 8, 21), and possibly Hk (18) are regulated by insulin levels. Both GSase and Hk are under allosteric regulation by G-6-P.

The coordinate regulation of the activity of several enzymes in this pathway by insulin has complicated efforts to assign a conventional rate-limiting step for glycogen synthesis. Correlations between the rates of GT and of glycogen synthesis or muscle glucose uptake have been interpreted as supporting GT control in transgenic mice overexpressing GLUT-4 (9, 36), in previously exercised mice overexpressing GLUT-4 (24), in glucose-clamped rats (11), and in the human forearm (4). However, the conclusion of transport control appears to contradict the extensive studies establishing that the activity of GSase correlates with the rate of muscle glycogen synthesis (3, 8, 40). The correlation between GSase activity and glycogen synthesis and the extensive regulation of GSase activity by phosphorylation have been the basis for the view of GSase as controlling the glycogen synthesis flux.

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enzymes in the pathway. Although these conditions cannot always be met experimentally, MCA has been applied successfully to determine flux control coefficients in a number of systems (12).

Recently, we have used MCA to reanalyze a study we performed using $^{13}$C and $^{31}$P NMR to measure insulin-stimulated muscle glycogen synthesis and G-6-P concentration in the muscle of healthy nondiabetic humans (26, 31). The analysis indicated that the activity of the combined steps of GT and HK (GT/Hk) determines the glycogen synthesis rate (31). We proposed that the role of the insulin-dependent alteration of GSase activity by phosphorylation was to regulate the concentration of G-6-P rather than to determine the rate of the pathway (31). The results of several studies of human and rat muscle performed under hyperinsulinemic conditions were shown to be consistent with these conclusions. However, it was necessary to combine results from different studies to show that the conditions necessary to apply MCA were met. The present in vivo NMR study was designed to allow calculation of the flux control coefficients of GT/Hk and GSase in the muscle glycogen synthesis pathway under hyperinsulinemic conditions in the rat. The synthesis rate of muscle glycogen was measured by $^{13}$C NMR during a hyperinsulinemic clamp at two glucose concentrations to alter selectively the GT activity. $^{31}$P NMR was used to verify that phosphorylated effectors of GSase other than G-6-P remained constant. The requirement for applying the analysis, that the phosphorylation state of GSase remain the same at both glucose levels, was established by direct assay. The application of MCA was extended from previous work (31) by incorporating the muscle glycolytic flux and using recently derived expressions that take into account the relatively large changes in plasma glucose concentration needed for the measurements (32, 33). The results support the conclusion that, under euglycemic and/or hyperinsulinemic clamped conditions, the increase in insulin-induced GT/phosphorylation activity is the major control point for determining the rate of muscle glycogen synthesis, whereas GSase has little control over the glycogen synthesis flux.

**MATERIALS AND METHODS**

*Protocol.* The experimental protocol is shown schematically in Fig. 1. Combined $^{13}$C/$^{31}$P NMR spectra were acquired during the baseline period ($t = -50$ min to $t = 0$) and up to 180 min during a glucose/insulin clamp. A primed, continuous infusion of somatostatin at 0.1 µg·kg$^{-1}$·min$^{-1}$ (Bachem) and insulin at 10 mU·kg$^{-1}$·min$^{-1}$ (NovoNordisk) was begun at $t = 0$ min. A primed, variable infusion of 20% wt/vol dextrose (100% [1-13C]dextrose) was begun at $t = 0$ min and adjusted to clamp the $\text{[glucose]}_\text{plasma}$ at $\sim 5.5$ mM (E; $n = 6$) within 40 min. Euglycemic conditions were maintained for 50 min, during which time $^{13}$C/$^{31}$P NMR spectra were acquired. At the end of this period, the infusion rate was adjusted to clamp the $\text{[glucose]}_\text{plasma}$ at $\sim 13.9$ mM (hyperglycemia; H; $n = 6$). After a 30-min period to allow the plasma glucose to stabilize, spectra were acquired during hyperglycemic conditions for 50 min. An additional animal, the clamp order was reversed (H followed by E) as a control. At the end of the hyperglycemic clamp, the gastrocnemius was resected and the muscle frozen in liquid $N_2$. The animals were euthanized by an intravenous dose of KCl. The muscles were stored at $-70^\circ$C until assayed for [glycogen] or GSase activity.

An additional group of animals was studied outside the magnet with unlabeled glucose under conditions identical to the E or H phases described above (non-NMR groups: E, $n = 5$; H, $n = 1$). The gastrocnemius was removed after the clamp period for determination of GSase activity.

*Animals.* Overnight-fasted male Sprague-Dawley rats, 260–340 g, were anesthetized with 100 mg/kg Na$_2$NaCO$_3$ thiopental. The rat was tracheotomized and ventilated on O$_2$ supplemented with room air. Catheters were inserted into the left jugular vein and right carotid artery for infusion and blood sampling, respectively. The skin over the calf and the knee was removed and the exposed muscle covered with plastic film. Brass pins were inserted under the patella and through the Achilles tendon, and the rat was fastened to a special Lucite holder for immobilization of the leg. For the NMR experiments, a coil was positioned directly over the exposed muscle. The animals were warmed throughout the experiment by means of a circulating water bath system. The animal protocol was approved by the Yale Animal Care and Use Committee.

Blood gases were monitored throughout on an ABL-30 analyzer (Radiometer, Copenhagen, Denmark), and the ventilation was adjusted to maintain O$_2$ at 90–120 mmHg and CO$_2$ at 35–45 mmHg. [Glucose]$_\text{plasma}$ was determined in duplicate by the glucose oxidase method in a Glucose Analyzer II (Beckman, Fullerton, CA). Glucose consumption was calculated from the average steady-state glucose infusion rate. [Insulin] was measured externally relative to human standards (VetResearch, White Plains, NY) from blood samples taken 10 min before the end of each clamp period.

*NMR spectroscopy.* NMR spectra were recorded on a 4.7 T horizontal bore Bruker Biospec spectrometer. The radio frequency probe consisted of two concentric coils: a 5-cm $^1$H-coil (200.4 MHz) for C-H decoupling and a 1.8 × 2-cm elliptical, double-turn, double-tune coil for $^{13}$C (50.4 MHz) and $^{31}$P (81.1 MHz). The inner coil was used for shimming on the H$_2$O frequency probe consisted of two concentric coils: a 5-cm$^1$H-coil (200.4 MHz) for C-H decoupling and a 1.8 × 2-cm elliptical, double-turn, double-tune coil for $^{13}$C (50.4 MHz) and $^{31}$P (81.1 MHz). The inner coil was used for shimming on the H$_2$O
with $^{31}P$ NMR acquisitions (TR = 4 s). For $^{13}C$ we used a 52-μs, 180° hard pulse at the coil center; for $^{31}P$ we used a 90° BIR4 adiabatic pulse (2.5 ms duration). Glycogen $^{13}C$-1 intensity was measured at 100.5 parts per million (ppm) in 40-Hz line-broadened spectra. For each block, 2,736 $^{13}C$ and 72 $^{31}P$ scans were obtained.

The procedure for quantitating the concentration of G-6-P and other phosphorylated metabolites from the $^{31}P$ NMR spectra (see Fig. 2) was the same as that described in Bloch et al. (1), in which the method was validated against traditional chemical extraction. Levels of phosphorylated metabolites were determined from $^{31}P$ spectra summed over 40 min during baseline H and E periods (Fig. 2, inset). The one-half area of the G-6-P peak (6.31 to 6.33 ppm relative to $P_i$) and the peak (0 to −1 ppm relative to $P_i$) were measured in the 40-Hz line-broadened baseline spectra, such as in Fig. 2. The peaks correspond to phosphomonoesters (PME, including G-6-P) $P_i$, phosphocreatine (PCr), γ-ATP, α-ATP, and β-ATP. The concentration of metabolites was determined by comparing the spectral areas to the area of the $P_i$ peak (crosshatched) from difference spectra (see Fig. 2) was the same as that described in Bloch et al. (1).

Calculations. The $[G-6-P]$ peak (6.31 to 6.33 ppm relative to $P_i$) and inset BIR4 adiabatic pulse (2.5 ms duration). Glycogen $^{13}C$-1 intensity was measured at 100.5 parts per million (ppm) in 40-Hz line-broadened spectra. For each block, 2,736 $^{13}C$ and 72 $^{31}P$ scans were obtained.

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In vitro assays. Total [glycogen] was determined by amyloglucosidase digestion of precipitated glycogen. The $[^{13}C]$glucose enrichments of glucose in extracted glycogen and plasma were determined by gas chromatography-mass spectrometry, as described previously (2). The fraction of G-6-P in the G-6-P independent form (GSase%I) was assayed from the incorporation of $[^{13}C]$glucose into glycogen by a modification of the method of Thomas (34) at 0 and 6.7 mM G-6-P; as described previously (2). The GSase velocity in muscle homogenates was assayed enzymatically in a "physiological" mixture containing 8.4% glycogen, 0.1 mM UDP-glucose (UDPG), 7.2 mM ATP, and 0, 0.2, 0.4, 0.8, and 1.2 mM G-6-P, corresponding to the in vivo range of [G-6-P].

Calculations. The [glycogen] at each time point was calculated as in Bloch et al. (2) from the increment in the $^{13}C$ spectra and the $[^{13}C]$glucose enrichment. The synthesis rates reported for E and H were calculated from the slope of the linear regression of the glycogen concentrations over the 50 min of the clamp periods.

MCA. The MCA description of the glycogen synthesis pathway used to calculate the flux control coefficients is described in the APPENDIX. The responsivity coefficient at euglycemia was calculated from the change in glycogen synthesis rate in going from E to H by use of the following expression (32), which takes into account the noninfinitesimal change in plasma glucose concentration

$$R_{GH} = \frac{[J_H - J_E] / [J_H]}{[G_H - G_E] / [G_H]}$$

where $J$ is the in vivo or in vitro glycogen synthesis flux, and $G$ is the concentration of plasma glucose. The in vitro and in vivo elasticities of GSase to G-6-P were calculated using


The term $J_{GT/Hk}$ is total muscle glucose uptake, which is the sum of muscle glycogen synthesis and glycolysis. For calculations, whole body glucose uptake was used to approximate muscle glucose uptake. Under the experimental conditions of hyperinsulinenia, studies have shown that the muscle is the major pathway of disposal of infused glucose. All elasticity values were calculated with the group averages.

A minimum estimate of the glycogen synthesis flux control coefficient for GT/Hk at euglycemia was calculated from the responsivity (30)

$$C_{GT/Hk}^{E} \geq R_{GH}$$

The relative glycogen synthesis flux control coefficients of GSase, GT/Hk, and glycolysis were calculated using the following expressions that are derived in the APPENDIX. The in vivo value for $\epsilon_{G-6-P}^{GSase}$ was used in these calculations. Elasticities and control coefficient values were calculated for $J_{glycolysis}/J_{GT/Hk}$ ranging from 0.14 to 0.50. This will maximize the estimate of $C_{GT/Hk}$ and minimize the estimate of $C_{GT/Hk}$.

$$C_{GT/Hk} = \frac{[J_H - J_E]}{[J_H - J_E]}$$

$$C_{GSase}^{E} = \frac{[J_H - J_E]}{[J_H - J_E]}$$

$$C_{GT/Hk}^{E} = \frac{[J_H - J_E]}{[J_H - J_E]}$$

All results are presented as means ± SE. Statistical significance was calculated using a two-tailed Student’s t-test for the paired data.

RESULTS

Glucose clamps. The concentrations of plasma glucose for individual animals during the E and H periods are shown in Table 1. Plasma glucose concentration increased 2.5 ± 0.03-fold, averaging 5.45 ± 0.05 and 13.8 ± 0.14 mM during the E and H clamps, respectively. The whole body glucose consumption increased to the same degree (2.8 ± 0.3-fold) as the plasma glucose consumption, averaging 123 ± 19 and 315 ± 17
During the E and H periods, respectively. The concentrations of plasma insulin were similar during the H and E periods [308 ± 65 vs. 216 ± 32 mU/ml, not significant (NS), P, 0.3].

Glycogen synthesis. The animals varied in the starting [glycogen], ranging from 13.5 to 31.8 mmol/kg wet wt, with the average 21.0 ± 2.5 mmol/kg wet wt. The total amount of glycogen synthesized during the experiment ranged from 15 to 41 mmol/kg wet wt. Individual curves of the increments in [glycogen] during the hyperinsulinemic clamp are shown in Fig. 3. The synthesis rate averaged 0.087 ± 0.012 and 0.24 ± 0.03 mmol·kg⁻¹·min⁻¹ (P < 0.01) during E and H, respectively. The rates of glycogen synthesis for individual animals are listed in Table 1. Although the rates of glycogen synthesis varied between animals, the relative rates during the E and H periods were similar in all animals (Table 1). The average glycogen synthesis rate increased in proportion with the [glucose] plasma (Fig. 4).

Concentrations of phosphorylated effectors. To verify the constancy of phosphorylated effectors of GSase, the concentrations of ATP and Pi were measured from different spectra under H and E conditions. The concentration of Pi did not change with plasma glucose level (1.4 ± 0.09 mM at E vs. 1.5 ± 0.1 at H, NS, P < 0.4). The concentration of ATP, which was assumed to be 7.2 mM, remained constant throughout the study. To assess the relative elasticities of GSase and GT/Hk to G-6-P, we measured the in vivo G-6-P concentration (Table 2). The baseline G-6-P concentration was 0.18 ± 0.02 mM. The G-6-P concentration did not increase significantly over baseline during the E portion of the clamp (0.22 ± 0.03 mM, NS, P < 0.001). The increase in [G-6-P] was smaller than the increase in glycogen synthesis rate between E and H, as shown in Fig. 5.

In vitro GSase%I and velocity as a function of [G-6-P]. To verify that the activity of GSase was not altered by the change in glucose concentration, the GSase%I was measured in vitro under standard conditions. No difference was found in GSase%I between E and H conditions (38 ± 3 vs. 44 ± 3%, NS, P < 0.2). To assess the regulation of GSase velocity by G-6-P, we measured the velocity of GSase extracted under E and H conditions as a function of [G-6-P] under physiological concentrations of Pi and ATP. The curve for

![Fig. 3. Glycogen increments over starting [glycogen] for 6 animals clamped at E followed by H. [Glycogen] for the 7th animal, for which the clamp order was reversed, is not shown. Values in text are averages of all 7 animals. Time 0 is start of glucose/insulin/somatostatin infusion.](http://ajpendo.physiology.org/)

![Fig. 4. Relative glycogen synthesis rates (means ± SE, gray bars) vs. [glucose] plasma (means ± SE, open bars). Rates increased 2.7-fold, whereas [glucose] plasma increased 2.5-fold, from E to H.](http://ajpendo.physiology.org/)
the velocity of the enzyme extracted under E and H as a function of [G-6-P] is shown in Fig. 6. The velocity in the in vivo range of [G-6-P] was determined from a linear regression. When the in vivo concentrations of G-6-P were used, the velocity for E at 0.22 mM G-6-P was found to be 14.6 mU·kg⁻¹ min⁻¹ and that for H at 0.40 mM G-6-P to be 26.1 mU·kg⁻¹ min⁻¹. The 1.8-fold change in in vitro velocity is significantly lower (P < 0.001, Z-test) than the 2.8 ± 0.2-fold change in glycogen synthesis flux.

Calculation of responsivity. The responsivity of glycogen synthesis flux (J) to plasma glucose was calculated from Eq. 2 and the measured fractional change in glycogen synthesis and [glucose]plasma for each animal (Table 1). The values of R50,J for individual animals ranged from 0.88 to 1.13, with an average R50,J = 1.1 ± 0.03 (Table 1). From this value and Eq. 6, the C50/GT/Hk was 1.1 ± 0.03 for the GT/Hk step.

Calculation of elasticities to [G-6-P]. The elasticity of GSase to [G-6-P] was calculated from the measured changes in the velocity of GSase in the in vitro assay and Eq. A9 to be e50,J[G-6-P] = 0.79. The in vivo elasticity of GSase to [G-6-P] was higher; e50,J[G-6-P] = 1.9. The e50,J[G-6-P] values were determined using Eq. 5 for a range of ratios of glycolysis to GT/Hk. For the range of ratios of 0.15 to 0.4, the e50,J[G-6-P] ranged from −0.185 to −0.556. Ratios <0.15 yielded values of e50,J[G-6-P] >0, which would correspond to activation of GT/Hk by product G-6-P.

Calculation of flux control coefficients from elasticities. From the calculated C50,J[G-6-P] and the in vivo e50,J[G-6-P] at each ratio of glycolytic and GT/Hk flux, the control coefficients for GT/Hk, GSase, and glycolysis were calculated (Table 3). The values were calculated for a glycolytic flux ranging from 0.15 to 0.4 times the flux through the GT/phosphorylation step. Throughout the range of glycolytic flux ratios, GT/Hk has the largest control coefficient for the glycogen synthesis flux, with a constant C50,J[GT/Hk] ~1.1 compared with C50,J[GSase] <0.5. The highest ratios of C50,J[GT/Hk] to C50,J[GSase] were found at a glycolytic flux equal to 0.15 of the GT/Hk step. The absolute value of the C50,J[GSase] was smaller than C50,J[glycolysis] at all ratios of Jglycolysis/JGT/Hk.

DISCUSSION

Because of the coordinate increases in the activity of GT, Hk, and GSase with insulin, it has long been difficult to assess the flux-controlling step(s) in the muscle glycogen synthesis pathway. In this study, we applied MCA to determine quantitatively the contributions of GT/Hk, GSase, and glycolysis to the control of this pathway. The synthesis rate of muscle glycogen was measured at two glucose concentrations during a hyperinsulinemic clamp to alter GT activity. A flux control coefficient of C50,J[GT/Hk] = 1.1 ± 0.03 was calculated for the GT/Hk step from the responsivity to glucose and Eq. 6, which indicates that the rate of glycogen synthesis is approximately proportional to the total activity of this step. An independent measure of C50,J[GT/Hk] based on the elasticities and the Branch Point Theorem yielded values comparable to the direct measure (Table 3). The measured in vitro and in vivo elasticity of GSase to G-6-P was high relative to the elasticity of GT/Hk measured in vivo, also indicative of minimal control by GSase over the muscle glycogen synthesis flux. Although glycolysis was not measured directly, it was shown not to affect the conclusion that GT/Hk is the major flux control point, whereas GSase exerts less control over the glycogen synthesis flux than does glycolysis. In the sections to follow, we discuss evidence
supporting the validity of the MCA approach used here and additional applications for the control of glycogen synthesis.

Validation of the MCA model of muscle glycogen synthesis. The primary assumption in applying MCA to calculate flux control is that the activity of GT/Hk may be altered (through plasma glucose concentration) without affecting the activity of GSase other than through a change in the concentration of G-6-P. A change in G-6-P concentration is allowable, because it is internal to the portion of the glycogen synthesis pathway being modeled (13, 31). The primary external effectors of GSase activity are ATP and P_i, which were measured by 31P NMR and found not to change between the E and H stages of the clamp. The constancy of ATP agrees with the findings in 31P NMR studies of human muscle during hyperinsulinemia (26, 27). In human studies, a small increase in [P_i] was measured during H relative to E under hyperinsulinemic conditions that was not observed in the rat muscle. The GSase activity may also be altered by phosphorylation. To assess whether this occurred, the GSase%I was measured and found to be the same under E and H conditions at the insulin level used in this study. This finding is in agreement with a previous study of human muscle (39), which found that, under high plasma insulin, GSase%I is independent of plasma glucose concentration.

Effects of muscle glycolysis on the calculated control coefficients. To calculate a flux control coefficient for GSase, it was assumed that the muscle glycolytic flux is independent of plasma glucose concentration under hyperinsulinemic conditions. If this is the case, then the elasticity of glycolysis to G-6-P is negligible, and the control coefficient of GSase may be directly calculated from the relative elasticities of GT/Hk and glycolysis. The independence of muscle glycolysis to glucose concentration is supported by several studies that estimated the constancy of muscle glycolysis either from measurements of whole body glucose oxidation (27, 40), for which muscle is the primary disposal pathway under hyperinsulinemic conditions, or from arteriovenous difference measurements across limbs (22). Under almost identical conditions of plasma insulin and glucose as used in the present study, Farrace and Rossetti (11) measured no change in glycolysis with glucose concentration.

Because glycolysis was not directly measured, the flux control coefficients were calculated for a range of glycolytic fluxes from 0.15 to 0.4, the rate of the flux through the GT/Hk combined reactions. The minimal value of 0.15 was used because e_{GT/Hk}^{G-6-P} ≤ 0 at ratios <0.145, which would indicate the improbable activation of Hk by product G-6-P. The maximum ratio of 0.5 was determined from the ratio of whole body glucose oxidation to whole body glucose uptake measured by Farrace and Rossetti (11), providing a maximum estimate of muscle glycolysis under these conditions of hyperinsulinemia because it neglects other tissues oxidizing glucose, such as the brain. More recently, Jucker et al. (15) measured directly the rates of rat muscle glycolysis and glycogen synthesis under conditions of euglycemic and hyperglycemic hyperinsulinemia using 13C NMR. They found that the muscle glycolysis flux did not change with plasma glucose level, which supports our assumption that the elasticity of glycolysis to G-6-P is close to 0. They measured a ratio for glycolysis of 0.3 to the total flux through GT/Hk under euglycemic conditions (15). Over the entire range of ratios (see Table 3), it was found that the flux control coefficient of GT/Hk is the highest for the pathway, whereas control by GSase remains much smaller. At higher values of the ratio of glycolysis to total GT/Hk flux, the glycolytic pathway exerts significant flux control for glycogen synthesis. The physiological significance of a high negative control coefficient of glycolysis may be to allow the muscle to rapidly shunt glucose away from storage as glycogen when an increase in energy is needed. Because of the low elasticity of glycolysis and GT/Hk to G-6-P, an increase in the glycolytic rate to meet the energy requirements of the muscle would shunt glucose away from storage as glycogen in a near 1:1 ratio. In contrast to the situation in the rat muscle, the relative rate of muscle glycolysis to muscle glycogen synthesis under hyperinsulinemic conditions in the human is low (27, 31), and this pathway will have a small flux control coefficient.

Comparison with other work. Several recent studies of muscle glycogen synthesis measured its rate at different plasma glucose concentrations under hyperinsulinemic conditions. We have calculated the flux control coefficient for GT/Hk from the results presented in these studies. The results of this calculation are shown in Table 4 (11, 21, 22, 26). The values, ranging from

Table 3. Calculated values of control coefficients for GT/Hk, GSase, and glycolysis for the flux for glucose through glycogen synthesis

<table>
<thead>
<tr>
<th>Ratio of Jglycolysis to J_{GT/Hk}</th>
<th>0.15</th>
<th>0.20</th>
<th>0.30</th>
<th>0.40</th>
<th>0.50</th>
</tr>
</thead>
<tbody>
<tr>
<td>e_{G-6-P}^{GT/Hk} (max)</td>
<td>-0.019</td>
<td>-0.126</td>
<td>-0.341</td>
<td>-0.556</td>
<td>-0.771</td>
</tr>
<tr>
<td>C_{GT/Hk}</td>
<td>1.16</td>
<td>1.15</td>
<td>1.15</td>
<td>1.14</td>
<td>1.10</td>
</tr>
<tr>
<td>C_{GSase}</td>
<td>0.011</td>
<td>0.077</td>
<td>0.204</td>
<td>0.328</td>
<td>0.448</td>
</tr>
<tr>
<td>C_{glycolysis}</td>
<td>-0.175</td>
<td>-0.231</td>
<td>-0.341</td>
<td>-0.448</td>
<td>-0.552</td>
</tr>
</tbody>
</table>

Calculations were made using the Branch Point Theorem and Eqs. 7, 8, and 9. GT/Hk, combination of steps of glucose transport and phosphorylation by hexokinase; GSase, glycogen synthase; J, flux. See text (Calculations) for explanation of other terms. It is assumed that e_{G-6-P}^{GT/Hk} = 1, yielding a maximal estimate for C_{GSase} and a minimal estimate for C_{GT/Hk}. The e_{G-6-P} is calculated with Eq. 5 for each $J_{glycolysis}/J_{GT/Hk}$ ratio. Calculated $e_{G-6-P}$ and in vivo $e_{G-6-P} = 1.9$ were used where needed in Eqs. A7, A8, and A9, and $e_{G-6-P}$...
literature values of \( \Delta S \) and \( \Delta G \) during hyperinsulinemia.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Control Coefficient ( C_{GT/Hk} )</th>
</tr>
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<tbody>
<tr>
<td>Rat (21)</td>
<td>1.74(4 ( \rightarrow ) 6 mM glucose)</td>
</tr>
<tr>
<td></td>
<td>1.25(6 ( \rightarrow ) 10 mM glucose)</td>
</tr>
<tr>
<td>Rat (11)</td>
<td>0.99</td>
</tr>
<tr>
<td>Human (22)</td>
<td>1.08</td>
</tr>
<tr>
<td>Human (27)</td>
<td>1.05</td>
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</table>

Coefficients were calculated, according to Eqs. 3 and 6 in text, from values taken from references shown in parentheses.

0.99 to 1.74, are consistent with the conclusion that GT/Hk exerts the majority of flux control in the muscle glycogen synthesis pathway. The values are generally greater than 1, which reflects the negative control coefficient of glycolysis. In the study of Jucker et al. (15), the change in glycogen synthesis rate and G-6-P with glucose level was similar to the change in the present study, which implies that the majority of control is exerted at GT/Hk. The elasticities of GT to G-6-P were estimated by inhibiting glycolysis with a free fatty acid (FFA) infusion, which led to a substantial decrease in total glucose uptake with only a small increase in G-6-P. From this result they concluded that the in vivo elasticity of GT/Hk is high (\( -2.18 \)), and they reported that the \( C_{GT/Hk} = 0.55 \pm 0.1 \) compared with a \( C_{GSase} = 0.30 \pm 0.06 \). The discrepancy from our finding of a low elasticity of GT/Hk may be reconciled by the subsequent finding that the activity of glucose transport is reduced during FFA infusion (10). In light of these findings, the reduction in total glucose uptake during FFA infusion was more likely due to a reduction in GT/Hk activity than a high elasticity of Hk, as proposed by the authors.

GT may exert the majority of flux control for the GT/Hk step. In the present analysis, the GT- and Hk-catalyzed reactions are treated as a single equivalent reaction, because the intracellular glucose concentration was not measured. The relative control exerted by the individual GT and Hk reactions may be determined from the intracellular glucose concentration. Several studies, including two recent \(^{13}\)C NMR studies (5, 28), have found that, under hyperinsulinemic conditions, intracellular muscle glucose is <1 mM, which is the precision of the measurement. The low concentration of intracellular glucose relative to plasma glucose and the high Michaelis-Menten constant of the transporters (\( \sim 20 \) mM glucose) (19) suggest that the glucose transport flux at euglycemia is insensitive to changes in intracellular glucose concentration. From this it may be concluded that transport is responsible for the major part of flux control. Studies that have found a correlation between unidirectional GT measured using nonmetabolizable glucose analogs and muscle glucose uptake (3, 6) are consistent with GT having a higher flux control coefficient than Hk.

Compartmentation. A complication in the calculation of the in vivo elasticities is the possibility of compartmentation of G-6-P leading to a different concentration in the environment of GSase than the whole tissue concentration measured by \(^{31}\)P NMR. This compartmentation may be between different fiber types or within the microenvironment of individual muscle cells. There is no effect of compartmentation on the calculated flux control coefficient of GT/Hk from the measured responsivity. The elasticity used in this calculation (31) is a minimum value that is assumed on the basis of the enzymatic properties of the isolated GT and Hk (31) enzymes. Furthermore, the concentration of the modulated effector (extracellular glucose) is not affected by compartmentation. For GSase to have a high control coefficient, its elasticity to G-6-P would have to be at least an order of magnitude lower than the values measured in vivo and in vitro. On the basis of in vitro kinetics of the enzyme, such a low elasticity would only occur at saturating concentrations of G-6-P, which at the GSase%I ratio measured in vitro would only occur at G-6-P concentrations well over 1 mM (25). This high local concentration is unlikely, because it defeats the ability to regulate the enzyme by phosphorylation, which alters the kinetics of GSase by shifting the G-6-P association constant of the enzyme.

The role of GSase in maintaining G-6-P homeostasis. The low flux control coefficient of GSase seems paradoxical, given the known increase in GSase activity with insulin concentration (7, 35, 39). The GSase%I measured in the present study is consistent with previous measurements under similar conditions and well above the activity measured under basal insulin (21, 25). An explanation we have proposed for the function of the insulin-dependent regulation of GSphosphorylation state is that it reduces the changes in G-6-P concentration required for the velocity of the enzyme to match the rate set by the GT/Hk steps. As shown by Roach and Larner (25) and others (23), under in vivo concentrations of ATP, the velocity of GSase has a sigmoidal relationship to %I at fixed G-6-P concentrations. The region of maximum sensitivity to %I at the in vivo G-6-P concentration of 200–400 \( \mu \)mol/kg is in the 30–40%I range achieved by insulin.

The high in vivo elasticity of GSase to G-6-P (Fig. 6) would further act to reduce the changes in G-6-P concentration needed to accommodate changes in GT/Hk velocity due to plasma glucose. A high elasticity to G-6-P was also measured in human muscle by use of \(^{31}\)P NMR to measure G-6-P concentrations (26). The high elasticity may be explained by the sigmoidal relationship between GSase velocity and G-6-P concentration under simulated in vivo conditions when GSase%I is <60% (25). We found the slope of the in vitro GSase velocity vs. G-6-P curve to be greater than unity in the in vivo G-6-P range (Fig. 6), which supports this explanation. However, within the accuracy of the in vivo measure of G-6-P concentration, other mechanisms cannot be ruled out.

Conclusions. Under hyperinsulinemic conditions, the combined reactions of GT and Hk primarily determine the rate of muscle glycogen synthesis in the rat. The use of different plasma glucose levels to modulate GT/Hk activity allows MCA to be applied to the pathway, because effectors external to the glycogen synthe-
sis pathway, such as ATP and the activity of GSase, were not affected by the glucose level. The low control coefficient of GSase is consistent with our previous proposal that the role of insulin stimulation of GSase dephosphorylation is for maintaining G-6-P homeostasis rather than controlling the flux (31). Because of the enhanced rate of glycolysis in rat muscle relative to human muscle, there may be a significant role for glycolysis in the control of the rate of glycogen synthesis. However, the primary control of the rate of muscle glycogen synthesis is exerted by the combined reactions of GT and Hk.

**APPENDIX**

In the MCA model of the glycogen synthesis pathway (30), the pathway is simplified into a two-step reaction. The combined reactions of GT and Hk convert extracellular glucose to G-6-P. Subsequently, G-6-P is converted by the combined reactions of glucose phosphate isomerase, UDP-glucose transferase, and glycogen synthase to glycogen. Because GSase is the only enzyme in the proximal reactions that changes activity with insulin, this series of reactions is referred to as GSase. The combination of sequential reaction steps into a single equivalent reaction has been shown to be valid, provided that substrates within a grouped series of reactions do not act as effectors outside of the group (13). For the muscle glycogen synthesis pathway, G-6-P is the only substrate that affects the activity of both the GT/Hk and GSase group of enzymes (38) and is therefore treated explicitly in the model.

The most direct method for determining the flux control coefficients of the enzymes in the glycogen synthesis pathway would be to vary the activity of each enzyme individually and to measure the resultant change in glycogen synthesis flux. For the case of muscle glycogen synthesis, it is not possible to directly alter the activity of GT/Hk without affecting the activity of GSase because of the coordinated regulation of these enzymes. In MCA, several methods have been developed to calculate flux control coefficients by modulating the in vivo velocity of an enzyme through variation of the concentration of either substrates or allosteric effectors. In this APPENDIX we describe the application of MCA to determine \( C'_{\text{GT/Hk}} \), \( C'_{\text{GSase}} \), and \( C'_{\text{glycolysis}} \) from the experimental data.

The glycogen synthesis flux control coefficient for the combined GT and Hk reactions is defined as

\[
C'_{\text{GT/Hk}} = \frac{(\delta J/J)/(\delta E_{\text{GT/Hk}})}{E_{\text{GT/Hk}}}
\]

(A1)

where \( E_{\text{GT/Hk}} \) is the total activity of the GT/Hk step, and \( J \) is the glycogen synthesis flux. The combined activity of GT/Hk is used in the analysis because the concentration of intracellular glucose cannot be directly measured. The sum of all the control coefficients is equal to 1 for an arbitrary metabolic pathway. The flux control coefficients of GT/Hk and GSase are related by the summation theorem of MCA (16)

\[
C'_{\text{GT/Hk}} + C'_{\text{GSase}} + C'_{\text{glycolysis}} = 1
\]

(A2)

The additional control coefficient is for glycolysis, which shunts G-6-P away from glycogen synthesis.

**Determination of \( C'_{\text{GT/Hk}} \) from the Measured Responsivity**

In the present study, we modulated the activity of the combined GT/Hk step through changing the level of the substrate, plasma glucose (\( G_s \)). The measured change in the glycogen synthesis flux was used to quantitate the responsivity (\( R_{J0}' \)) of the pathway flux, where

\[
R_{J0}' = \frac{(\delta J/J)/(\delta G_s/G_0)}{G_s} \quad (A3)
\]

The responsivity yields an estimate for \( C'_{\text{GSase}} \) at euglycemia from the relationship (17)

\[
R_{J0}' = \kappa C'_{\text{GT/Hk}} \quad (A4)
\]

It has been shown for the combined reactions of GT and Hk that \( \kappa \) is not 1 (31), so that a minimum estimate of the flux control coefficient of GT/Hk is given by

\[
C'_{\text{GT/Hk}} \geq R_{J0}' \quad (A5)
\]

Equation A5 was used to calculate \( C'_{\text{GSase}} \) from the experimental measurement of the responsivity (Eq. 3).

**Determination of \( C'_{\text{GT/Hk}} \) and \( C'_{\text{GSase}} \) from the Measured Elasticities to G-6-P and the Branch Point Theorem**

The relative control of GT/Hk and GSase was determined from the sensitivity of the velocity of these reactions to the change in G-6-P concentration. This sensitivity, termed “elasticity” (\( \varepsilon \)), is defined as the fractional change in enzyme velocity (\( V \)) for a fractional change in the level of an effector, \( Z \)

\[
\varepsilon = \frac{(\delta V/V)/(\delta Z/Z)}{} \quad (A6)
\]

This relationship is valid at the in vivo substrate and effector levels. The value may be quantitated in vivo, or in vitro if assayed in the presence of all effectors at their in vivo levels. On the basis of the connectivity theorem of MCA (16), the control coefficients for GT/Hk and GSase and glycolysis are related by the elasticities of these pathways to G-6-P:

\[
C'_{\text{GT/Hk}} \cdot \varepsilon_{\text{G-6-P}} + C'_{\text{GSase}} \cdot \varepsilon_{\text{G-6-P}} + C'_{\text{glycolysis}} \cdot \varepsilon_{\text{G-6-P}} = 0 \quad (A7)
\]

The reaction that is more sensitive to changes in G-6-P has lower flux control for glycogen synthesis. Because of the low elasticity of glycolysis to G-6-P, the above relationship holds, even though the pathway branches at G-6-P, as will be shown.

A third relationship among the control coefficients of GT/Hk, GSase, and glycolysis is provided by the branch point theorem of Fell and Sauro (13)

\[
C'_{\text{GT/Hk}} \cdot J_{\text{GT/Hk}} + C'_{\text{glycolysis}} \cdot J_{\text{glycolysis}} = 0 \quad (A8)
\]

The terms \( J_{\text{GT/Hk}} \) and \( J_{\text{glycolysis}} \) are the fluxes through the GT/phosphorylation-combined reactions and glycolysis, respectively. Equations A2, A7, and A8 were solved for expressions for \( C'_{\text{GT/Hk}} \), \( C'_{\text{GSase}} \), and \( C'_{\text{glycolysis}} \) in terms of the elasticities and the fluxes \( J_{\text{GT/Hk}} \) and \( J_{\text{glycolysis}} \)

\[
C'_{\text{GT/Hk}} = -\frac{C'_{\text{glycolysis}} J_{\text{GT/Hk}} G_s}{J_{\text{glycolysis}} G_0 - J_{\text{glycolysis}} J_{\text{GT/Hk}} G_s} \quad (A9)
\]

\[
C'_{\text{GSase}} = \frac{-J_{\text{glycolysis}} J_{\text{GT/Hk}} C'_{\text{GT/Hk}}}{(G_s J_{\text{glycolysis}} J_{\text{GT/Hk}} G_0 - J_{\text{glycolysis}} J_{\text{GT/Hk}} G_s)^{-1}} \quad (A10)
\]

\[
C'_{\text{glycolysis}} = \frac{1 - (G_s J_{\text{glycolysis}} J_{\text{GT/Hk}} G_0 - J_{\text{glycolysis}} J_{\text{GT/Hk}} G_s)^{-1}}{(J_{\text{glycolysis}} J_{\text{GT/Hk}} G_0 - J_{\text{glycolysis}} J_{\text{GT/Hk}} G_s)^{-1}} \quad (A11)
\]

**In Vivo Elasticities of GT/Hk, GSase, and Glycolysis to G-6-P**

Relative flux control was determined from the ratio of the in vivo elasticity of GSase to the in vivo elasticity of GT/Hk to G-6-P changes (Eq. A7). The in vivo elasticity of GSase was calculated using Eq. 4 in the text. Calculation of the elasticity of GT/Hk is complicated by the change in the external sub-
strate, plasma glucose. Writing the velocity of the GT/Hk step as a total differential in $G_o$ and $G-6-P$ gives

\[ \delta J_{\text{GT/Hk}}/J_{\text{GT/Hk}} = \epsilon_{G_o}^{\text{GT/Hk}} \delta G_o/G_o + \epsilon_{G-6-P}^{\text{GT/Hk}} (\delta G-6-P/G-6-P) \]  

(A12)

Because the GT/Hk reaction feeds both glycogen synthesis and glycolysis, the flux $J'$ is the sum of these pathways. Equation A12 may be solved for $\epsilon_{G-6-P}^{\text{GT/Hk}}$, yielding

\[ \frac{\epsilon_{G-6-P}^{\text{GT/Hk}} (\max)}{\delta J_{\text{GT/Hk}}} = \frac{\delta J'}{J_{E}^{\text{max}} - \epsilon_{G_o}^{\text{GT/Hk}}} \]  

(A13)

We have shown previously that, as a result of the kinetic properties of GT and Hk, the maximum value for $\epsilon_{G-6-P}^{\text{GT/Hk}}$ is 1 (31). Because $\epsilon_{G-6-P}^{\text{GT/Hk}} \leq 0$ because of product inhibition of the Hk reaction, and both the fractional increase of G-6-P concentration ($\delta G-6-P/G-6-P_E$) and total glucose uptake ($\delta G_o/G_o_E$) were positive at the higher plasma glucose concentration, then the maximum negative value of $\epsilon_{G-6-P}^{\text{GT/Hk}}$ is given when $\epsilon_{G-o}^{\text{GT/Hk}}$ is set to its maximum value of 1

\[ \epsilon_{G-6-P}^{\text{GT/Hk}} (max) = \frac{(\delta J_{\text{GT/Hk}}/J_{\text{GT/Hk}} - \delta G_o/G_o_E)}{\delta G-6-P/G-6-P_E} \]  

(A14)

The value $\epsilon_{G-6-P}^{\text{GT/Hk}}$ was calculated for a range of $J_{\text{glycolysis}}/J_{\text{GT/Hk}}$ values (0.5–0.15) and used in Eq. A4.

Effect of Glycolysis

Previously, glycolysis was not included in the MCA of the glycogen synthesis pathway, because in nondiabetic human subjects it is a small fraction of total glucose uptake under hyperglycemic hyperinsulinemic conditions (31). Under the experimental conditions in the rat muscle, glycolysis may account for up to one-half of the glucose uptake at euglycemic conditions and is therefore included in the control analysis.

According to measurements by Farrace and Rossetti (11), the rate of muscle glycolysis does not change with plasma glucose during hyperinsulinemia. Therefore, $e_{\text{glycolysis}}^{\text{glycolysis}}$ was set to 0 in Eq. A9 for calculation of the flux control coefficient of GSase. If this assumption is not correct, the $e_{\text{glycolysis}}^{\text{glycolysis}} > 0$, the effect would be that our assumption leads to an overestimation of the control coefficient of GSase, because $e_{\text{glycolysis}}^{\text{glycolysis}}$ is positive, whereas $\epsilon_{G-6-P}^{\text{GT/Hk}}$ is negative. The calculations of flux control coefficients (Eqs. A9, A10, and A11) were performed for a range of $J_{\text{glycolysis}}$ from 0 to 0.5 $J_{\text{GT/Hk}}$.

REFERENCES


