Control of Glucose Utilization in Working Perfused Rat Heart*

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Metabolic control analyses of glucose utilization were performed for four groups of working rat hearts perfused with Krebs-Henseleit buffer containing 10 mM glucose only, or with the addition of 4 mM D-β-hydroxybutyrate/1 mM acetoacetate, 100 mM insulin (0.85 unit/ml), or both. Net glycogen breakdown occurred in the glucose group only and was converted to net glycogen synthesis in the presence of all additions. The flux of [2-3H]glucose through P-glucoisomerase (EC 5.3.1.9) was reduced with ketones, elevated with insulin, and unchanged with the combination. Net glycolytic flux was reduced in the presence of ketones and the combination.

The flux control coefficients were determined for the portion of the pathway involving glucose transport to the branches of glycogen synthesis and glycolysis. Major control was divided between the glucose transporter and hexokinase (EC 2.7.1.1) in the glucose group. The distribution of the control was slightly shifted to hexokinase with ketones, and control at the glucose transport step was abolished in the presence of insulin. Analysis of the pathway from 3-P-glycerate to pyruvate determined that the major control was shared by enolase (EC 4.2.1.1) and pyruvate kinase (EC 2.7.1.40) in the glucose group. Addition of ketones, insulin, or the combination shifted the control to P-glycolate mutase (EC 5.4.2.1) and pyruvate kinase.

These results illustrate that the control of the metabolic flux in glucose metabolism of rat heart is not exerted by a single enzyme but variably distributed among enzymes depending upon substrate availability, hormonal stimulation, or other changes of conditions.

With the demonstration that the concentration of intracellular glucose was very low in skeletal muscle, it was first suggested that insulin might act primarily by increasing glucose transport into the cell (1). The evidence that administration of insulin increased the galactose space in eviscerated dogs (2) confirmed that a major action of insulin was to increase intracellular hexoses in insulin-sensitive tissues. It was subsequently shown that glucose transport was mediated in muscle by a carrier-facilitated process (3). In retrograde perfusion of isolated rat hearts, administration of insulin did not alter the apparent $K_m$ of 3-O-methyl glucose transport in the forward direction but rather increased its apparent $V_{max}$ from near 1 μmol/min/g to 12 μmol/min/g while at the same time decreasing the $K_m$ in the reverse direction from 7 to 3 mM (4). The further finding that hearts perfused in the retrograde manner had unmeasurable [Glc]$^\text{t}$ at concentrations of extracellular glucose [Glc]$^\text{t}$ from 2 to 16 mM contributed to the belief that [Glc]$^\text{t}$ was very low and that, therefore, glucose transport was rate-limiting for reactions utilizing glucose (5). However, in the same study it was also observed that [Glc]$^\text{t}$ was about 1.5 mM when hearts were perfused in the working mode, provided that extracellular glucose was elevated to the mildly hyperglycemic concentration of 16 mM and that the atrial pressure was maintained below 5 cm H$_2$O to limit the work-related requirement for substrate. Under very slight differences in experimental conditions, therefore, limitations imposed on a particular enzyme step were altered so that this step was no longer rate-limiting. It is, therefore, to be expected that a step that exerts a dominant effect upon flux under one set of conditions, such as insulin deprivation, may exert a quantitatively different effect upon flux under different experimental conditions (6, 7).

In contrast to the view that glucose transport is the "rate-limiting" or "pacemaker" reaction of glucose utilization, an alternative view has been expressed that many, if not all, of the enzymes of glycolysis have activities similar to the rate of glycolysis itself and that one or another of these enzymes may control flux under different conditions. Decreases of only 33% in the activity of an enzyme, P-glycolate isomerase, thought to operate in vivo very close to near-equilibrium, have been reported (8) to decrease significantly the ratio of [products] to [reactants] (1') measured in tissue and at the same time to cause a proportionate decrease in the rate of complex carbohydrate synthesis.

One way to deal quantitatively with the distribution of flux under different conditions is to determine the rate of flux through a metabolic pathway exerted by individual enzymatic steps or groups of enzymatic steps that have been formalized in the concept of control strength (9), which...
was subsequently termed flux control coefficient (10). Flux control coefficients \( (C) \) are defined as the fractional change in flux resulting from an infinitesimal fractional change in enzyme activity. The flux control coefficient can be determined indirectly by first calculating the elasticity \( (\varepsilon) \), the fractional change in the net rate of a reaction catalyzed by a particular enzyme brought about by infinitesimal fractional change in the concentration of its substrate or product (Equation 6). The degree of control exerted by each enzyme step can be determined using bottom-up analysis (9, 11). In a steady state, net rate \( (v) \) can be calculated from known kinetic and thermodynamic parameters using the Haldane equation (Equation 4) (12) to obtain the elasticity (Equation 8). The degree of control exerted by groups of enzymes can also be determined using top-down analysis (11, 13, 14). In this paper, the method of analysis is closest to that of Groen et al. (15). Flux can be varied in different ways: the activity of a particular enzyme may be decreased by inhibitor titration (18) or by the selection of mutants with lower activity of a specific enzyme (8); enzyme activities may be increased by addition of pure enzyme to a tissue homogenate (17) or by genetically engineered overexpression (18). Other methods to formalize analysis of flux control coefficients have also been developed (19–25).

In this study we were known to increase glucose transport into heart muscle (4, 26). Glucose transport was later shown to result from the translocation of Glut4 from the endoplasmic reticulum to the plasma membrane (27–31). Because insulin administration increases rapidly the number of Glut4 molecules located within the plasma membrane, glucose transport into the interior of perfused working rat heart increases as well. However, with the provision of a preferred fuel such as ketone bodies (32–34) for energy production, the transport of glucose to supply the energy requirements for the heart should decrease. We have used these two approaches: first, increasing the activity of the glucose transporter by a saturating dose of insulin (100 mM); and second, reducing the need for glucose transport by supplying 4 mM sodium \( \beta\)-hydroxybutyrate and 1 mM sodium acetocetate as an alternative energy source. Using these strategies, we have determined for the first time in one set of data the concentrations of the intermediate metabolites, the kinetic constants of the enzymes of glucose metabolism, the values of equilibrium constants \( (K') \) for intracellular conditions of \( pH \) and free \( [Mg^2+] \), and the flux of the pathway. With this information we have examined the effects of different physiological manipulations on the control of flux exerted by the enzymatic steps of glycolysis and glycogen metabolism of perfused working rat heart.

**EXPERIMENTAL PROCEDURES**

**Preparation and Perfusion of Rat Hearts**

450–500-g male Wistar rats (Charles River Laboratories, Wilmington, MA) fed ad libitum were given 50 mg/kg of sodium pento-barbital/kg of body weight intraperitoneally (Signs). Hearts were removed and perfused in a nonrecirculating hemoglobin-free system (35) as previously described (36). Briefly, following rapid excision, hearts were perfused in a retrograde manner (a modified Langendorff technique [37]) with modified Krebs-Henseleit buffer, \( pH \) 7.4, containing 1.2 mM \( P_i \), free \( [Mg^2+] \) of 0.5 mM, free \( [Ca^2+] \) of 1.07 mM, and 10 mM glucose for \( \sim 15 \) min. During this period, the venae cavae and the pulmonary veins were ligated, and the pulmonary artery was catheterized to collect the effluent of the coronary sinus and the right ventricular thebesian veins. Hearts were then switched to the working mode (35) with a 10 mM \( H_2O \) preload and 80 mm Hg (108 cm \( H_2O \)) afterload for 15 min for a period of stabilization. During the succeeding 30 min, hearts were perfused with one of four buffer solutions containing: 1) 10 mM glucose; 2) 10 mM glucose plus 1 mM sodium acetocetate and 4 mM sodium \( \beta\)-hydroxybutyrate (Sigma); 3) 10 mM glucose plus 1 \( \mu M \) insulin (100 units or 4 mg per ml) (Novo-Squibb, Princeton, NJ), a dose of insulin found in skeletal muscle preparations to give maximal insulin response in vivo (38); or 4) 10 mM glucose plus the combination of ketone bodies and insulin. During this period, a number of parameters of cardiac function were measured as previously described (36): aortic flow, coronary flow, left ventricular \( dP/dt \) (Gould G4615-71, Valley View, OH), systolic aortic pressure (Spectramed P41400, CA), diastolic aortic pressure and aortic pressure, and left ventricular systolic pressure (Millar SPR477, Houston TX). At the end of the 30-min period, hearts were clamp-free-clamped to a thickness of <2 mm, submersed under liquid nitrogen, and the atrium and any adherent perufused were removed with a dental drill. Extracts for metabolite and ion analyses were made as previously described (36), except that the extracts for Fru-2,6-P, analysis were prepared in 0.06 M NaOH (39) and those for glycogen were prepared by heating the tissue samples in 0.5 M NaOH at 100 °C for 15 min to destroy the glucose and solubilize the glycogen.

**Enzyme Measurements**

For most enzyme determinations, heart tissues were minced with scissors; homogenized at low speed on a Potter-Erleheim glass homogenizer in 10–20 volumes of a 1:1 \( H_2O \)-glycerol mixture containing 10 mM \( K_2HPO_4, KH_2PO_4, pH \) 7.4, 20 mM imidazole, \( pH \) 7.4, 5 mM mercaptoethanol, 0.5 mM EDTA, and 0.02% bovine plasma albumin; and used without centrifugation. Where two substrates were involved, the \( K_c \) of the first substrate was determined in the presence of saturating concentrations of the second substrate. To preserve the interconvertible forms of glycogen synthase (EC 2.4.1.11) and phosphorylase (EC 2.4.1.1) separate homogenates were prepared in 20 mM imidazole buffer, \( pH \) 7, 0.5 mM dithiothreitol, 5 mM EDTA, and 0.02% bovine plasma albumin. Other enzyme activities were determined at 37 °C using fluorometric procedures measuring the oxidation or reduction of pyridine nucleotides (40). The basic reagent was 10 mM \( K_2HPO_4, KH_2PO_4 \) buffer, \( pH \) 7.2, 20 mM imidazole \( RCl \), \( pH \) 7.2, 150 mM KCl, and 5 mM MgCl_2 substrates, auxillary enzymes, and cofactors were added as necessary. The kinetics of all of the enzymes of glycolysis were measured as well as the kinetics of glycogen synthase, phosphorylase, \( P\)-glucosamutase (EC 5.4.2.2), and glucose-6-phosphatase (EC 3.1.3.9). The analyses were conducted at high dilution by fluorometric procedures that minimize disturbing side reactions. Appropriate blanks were included to correct for nonspecific reactions. More than one form of an enzyme might be present; the numbers represent an average value in the crude tissue; the homogenates were not purified in any manner and thus should represent as closely as possible the activity of the tissue itself. In specific instances, the reverse reactions were measured in order to do metabolic control analysis (Tables V, VI, and VII). Certain equilibrium and kinetic constants were obtained from the literature as noted in Tables IV and V (41–57).

**Analytical Measurements**

Intra- and extracellular water spaces were measured with perfusate containing 1 \( \mu Ci/ml \) \( \text{H}_2O \) (DuPont NEN) and 0.05 \( \mu Ci/ml \) [\( ^l\]C]mannitol (ICN Biochemicals, Costa Mesa, CA) for 5 min as previously described (36). Unless otherwise stated, glycolytic intermediates were measured using a ratio fluorometer (Optical Technology Devices, Inc., Elmsford, NY) by established methods (40). Fru2,6-P_2 was measured by a fluorometric adaptation of the method of Rose and Liebowitz (58). Fru-2,6-P_2 was measured by a fluorometric adaptation of a published enzymatic method (39). CAMP was measured by radioimmunoassay (DuPont NEN). Values for metabolites, nucleotides, and cofactors are given as micromoles per milliliter of intracellular water; to convert micromoles per gram wet weight, to micromoles per milliliter of intracellular space, the values were multiplied by 2.086 as determined by space measurements.

**Metabolic Flux**

The rate of glucose utilization was determined with high pressure liquid chromatography purified (2-HGlucone (DuPont NEN). Tritiated glucose (0.75 mCi) was added to 200 ml of perfusate (375 \( \mu Ci/mmol \)). The coronary outflow was collected during a timed interval, usually 2 min. The phosphorylated intermediates and glucose were removed by passing the sample over formate and borate Dowex 50 columns in sequence (59). The tritiated water remaining was a measure of the flux through P-glucosimerase; no correction was made for incomplete equilibration at this step (60). The removal of the labeled glucose and intermediates was confirmed by evaporating a portion of the effluent from the column; less than 0.1% of the counts remained. The rates of glycolysis or glycogenesis were calculated from the Aglycogen as gluco-ey units from the end of the initial perfusion of 15 min to the end of the 30-min experimental treatment (Fig. 1).
Calculations

Statistical analyses of the significance of the difference between means were calculated using a Mann-Whitney U test (Stat-View-4, Abacus Concepts, Inc., Berkeley, CA). Cytoplasmic [P] was determined by 31P NMR; the cytoplasmic pH was taken from the shift of the intracellular P from phosphocreatine, and the cytoplasmic free [Mg2+] was calculated using the measured [ATP]/[ADP] ratio (61) (Table II). Cytoplasmic [ATP]/[ADP] ratios were calculated as previously described (36). All equilibrium constants were corrected, where appropriate, for the pH and free [Mg2+] by methods previously described (62) using the equilibrium constants that had been determined under conditions approximating the intracellular environment. The values for [GAP], [Fru-1,6-P2], and [1,3-P2-glycerate] are calculated from the equilibrium constant of triose-P isomerase (EC 5.3.1.1), aldolase (EC 4.1.2.13), and 3-P-glycerate kinase (EC 2.7.2.3) using the individual values of measured metabolites. All other calculations were made correcting for intra- and extracellular volumes (36). Changes in metabolite levels are presented as proportionate change, which is equal to n (experimental value/control value); n = 1 when the experimental > control, and n = -1 when the experimental < control, thus assigning equal value to increases and decreases (63). Cardiac hydraulic work was calculated as follows,

\[
\text{cardiac output (mL/min/g wet weight)} = \frac{\text{cardiac output (mL/min) × aortic pressure (mm Hg)}}{\text{left ventricle weight (g wet weight)}} \times 101,325 \text{ (Nm}^2) \times \frac{760 \text{ (mm Hg)}}{1 \text{ atm}}
\]

where 1 atm = 760 mm Hg = 101,325 newtons/m² (Nm²).

Method of Calculation of Metabolic Control Parameters

A variable property of a system will respond to a variation of some parameter, for example, metabolic flux will respond to changes in enzyme activity or metabolite concentration. A simple model is shown below.

\[ J = \frac{\text{Substrate } [X_i] \times \text{Product } [X_j]}{E_i} \]  

where i is number of the step, 1- (in this model, i = 1); [X] is concentration of the metabolite ([X] = [X_i], concentration of the substrate, [S_i], [X_j] = [X_j], concentration of the product, P_i); u_i is rate of conversion of substrate to product; U_i is rate of conversion of product to substrate; u/G_i is net rate of an individual enzyme step; and J is flux through the pathway of the system (in this one-step model J = u_i - u_j; and E_i is enzyme catalyzing step i denoted by the subscript.

1. Michaelis-Menten Initial Rate Equation with One Substrate or One Product (64)

\[ u_i = \frac{V_{max}[1 + K_{m,p}[S]]}{1 + [K_{m,p}]/[P]} \]

where K_m,p is Michaelis constant (K_m) of [S], forward direction; K_m,p is Michaelis constant (K_m) of [P], reverse direction; V_max is maximum velocity of an enzyme step in the forward direction; and V_max is maximum velocity of an enzyme step in the reverse direction.

2. Law of Microscopic Reversibility, Haldane Relationship, and Steady-state Rate Equation with One Substrate and One Product. Assuming Michaelis-Menten Kinetics (12)

At equilibrium,

\[ u_i = u_j \]  

\[ V_{max}/K_{m,p}[S] - [P]/K_{m,p} \]  

\[ 1 + [S]/K_{m,p} + [P]/K_{m,p} \]

where K is the equilibrium ratio of [P] to [S] determined in vitro under specified conditions.

3. Flux Control Coefficient

The flux control coefficient (C) is the fractional change in the flux of the system due to an infinitesimal fractional change in the rate of enzyme catalytic activity (δε/ε) in step i.

\[ C_i = \lim_{\delta \epsilon \rightarrow 0} \frac{\delta J}{\delta \epsilon} = \frac{\delta J}{\delta \epsilon} \]

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C can be calculated in two ways. In the direct method, flux is measured and the enzyme concentration is titrated by the addition of enzyme or inhibitors and does not require the calculation of elasticity (17, 65-67). In the indirect method, elastistic of the steps must first be determined (see step 4 below), and C can be calculated from these data (9, 15, 19, 20).

4. Elasticity

The elasticity (ε) of an enzyme is the fractional change in the net rate for an individual reaction (δε/δε), caused by an infinitesimal fractional change of metabolite concentration (δX/[X]) in step i. In this model, X_i is the substrate and X_j is the product of E_i (for review see Ref. 23).

\[ \varepsilon_{X_i}^{\text{(substrate)}} = \lim_{\delta \epsilon \rightarrow 0} \frac{\delta \epsilon_{[S]}/\epsilon_{[S]}}{\delta \epsilon / \epsilon} = \frac{\delta \epsilon_{[S]}}{\delta \epsilon / \epsilon} \]

\[ \varepsilon_{X_j}^{\text{(product)}} = \lim_{\delta \epsilon \rightarrow 0} \frac{\delta \epsilon_{[P]} / \epsilon_{[P]}}{\delta \epsilon / \epsilon} = \frac{\delta \epsilon_{[P]}}{\delta \epsilon / \epsilon} \]

4a. Calculation of ε from Change in Flux

\[ \varepsilon_{\text{change}} \sim \Delta \text{ln}[S], \quad \varepsilon_{\text{change}} \sim \Delta \text{ln}[P] \]  

This equation can be regarded as the finite approximation to the elasticity definition. To calculate ε by this method one needs measurements of net rate (Table I) and substrate concentrations (Table II). The above relationship (Equation 7) allows estimation of the block elasticity if the following conditions are met: 1) the flux (J) through a block of the reaction is equal to the net rate (u_i) of the step, 2) the flux is dependent on the concentration of only one metabolite outside of the block, and 3) the flux is changed by a perturbation in some other part of the metabolic pathway that causes a change in [S].

4b. Calculation of ε from Kinetic Data—If a steady state prevails and if the tissue reactions follow Michaelis-Menten kinetics, the net rate of the steady-state equation (Equation 4) can be substituted into Equation 6. The derivative of Equation 6 yields Equation 8. To calculate ε from the kinetic parameters of the enzymes one needs the following: (a) the K_i and V_max of forward and backward direction of each enzyme involved and the K_i and K_j of other substances that alter the kinetic parameters (Table V); (b) the concentration of the substrates ([S_i] and products ([P]) of each reaction (Table II); and (c) the equilibrium constant (K_i) of the reaction under appropriate conditions of temperature, ionic strength, pH, and free [Mg2+] under which the reactions are occurring (Table IV); and (d) the architecture of the pathway, that is whether it is linear or branched, and the ratio of fluxes at the branch points (Table I and Equation 11).

\[ \varepsilon_{X_j}^{\epsilon} = \frac{1}{1 - \Gamma K_j^{-1}} - 1 \]

\[ \varepsilon_{X_i}^{\epsilon} = \frac{1}{1 - \Gamma K_i^{-1}} - 1 \]

The expressions u/V_max and u/V_max represent the fractional saturation of the enzyme with [S] and [P], respectively (to the extent that K_m,p and K_m,p approximate true dissociation constants).

5. Summation Theorem

The sum of all flux control coefficients of any chosen linear flux (J) for all of the n enzymes in a metabolic sequence is 1.

\[ \sum_{i=1}^{n} C_i = 1 \]

where n is the number of steps in the sequence chosen.

6. Connectivity Theorem

The flux control coefficients are related to the elasticities of all en-
zymes that respond to the concentration of a single metabolite \( [X] \).

\[
\sum_{i=1}^{n} C_{i}^{i} v_{i} = 0 \quad \text{(Eq. 10)}
\]

7. **Branch Point Theorem**

At a branch point under steady-state conditions, the sum of the flux control coefficients of enzymes in the branches is equal to the ratio of flux through the branches.

\[
\sum_{i=1}^{n} C_{i}^{i} v_{i} = \frac{v_{i}}{v_{i,\text{max}}} \quad \text{in glucose group}
\]

\[
\sum_{i=1}^{n} C_{i}^{i} v_{i} = 1 \quad \text{in other groups}
\]

for the glucose group specifically,

\[
(C_{\alpha}^{\alpha} + C_{\beta}^{\beta}) v_{\alpha} = v_{\beta} \quad \text{in glucose group}
\]

and for the other groups,

\[
(C_{\alpha}^{\alpha} + C_{\beta}^{\beta}) v_{\alpha} = v_{\beta} \quad \text{in other groups}
\]

where \( \alpha, \beta, \gamma \) are branched steps in step \( i \).

8. **Matrix Method of Control Analysis**

\[
E = M, \quad C = E^{-1} M \quad \text{(Eq. 12)}
\]

where \( E \) is the elasticity matrix; \( C \) is the flux control coefficient matrix; and \( M \) is the matrix that defines the relationships between the elements of \( E \) and the flux control coefficients in \( C \).

### Application of Metabolic Control Analysis

We performed “bottom-up” (11, 23) and “top-down” (11, 13, 14) analyses. Bottom-up analysis considers individual enzyme steps with regard to their elasticities. In contrast, top-down analysis considers groups of enzymes, using group elasticities. In our applications, the differences between top-down and bottom-up analyses are: 1) top-down analysis applies to the whole of glucose metabolism, i.e., the effects of reactions beyond the block analyzed are included; 2) because different block elasticities were derived by taking different pairs of the experimental sets, there is a single result from the top-down method, which is an approximate average over the states considered. Fuller explanations as models are given below.

1. **Model of Bottom-up Analysis**

\( \epsilon \) was determined for the individual enzyme steps of the branched pathway from glucose transport to glycogen metabolism and \( P \)-glucosomerase step (Scheme 1) and also for the steps of the terminal linear pathway of glycolysis from 3-P-glycerate kinase to pyruvate kinase (Scheme 2) using the data from Tables II–V. For each pathway analyzed, \( \Sigma \epsilon_{i} = 1 \).

1. **Glucose Transport to the Branches of Glycogen Metabolism and \( P \)-Glucosomerase Step**—Scheme 1 is a model of glycogen metabolism and illustrates both glycogenolysis and glycogen synthesis. The activity of \( G \)-6-P dehydrogenase (EC 1.1.1.49) in the heart was \( 10^{2} \)-lower than that of \( P \)-glucomutase and \( P \)-glucosomerase (Table V), and the oxidative portion of the hexose monophosphate pathway was, therefore, ignored. In the presence of glucose alone, there was net phosphorylation of glycogen (shown as dashed arrow) and the net rate of the \( P \)-glucomutase reaction was in the direction of \( G \)-6-P formation; \( J_{G} = J_{G}^{-} \), \( J_{G} = 0 \). The value of glycogen synthase was not considered. In the presence of ketones and/or insulin, there was net glycogen synthesis, and the net rate of the \( P \)-glucomutase reaction was in the direction of \( G \)-1-P formation (shown as a solid arrow); \( J_{G} = J_{G}^{-} \), \( J_{G} = 0 \). In these cases, phosphorylation was not considered. Elasticities were calculated using kinetic data (Equation 8).
Ketone Bodies and Insulin Action on Glucose Utilization

In the glucose and ketones groups, \( \Gamma \) for the glucose transport step was far from equilibrium; intracellular glucose was low but several times higher than the \( K_c \) of hexokinase for glucose, so the use of glucose by hexokinase would be favored. The elasticity calculation does include a contribution for the reversibility in the term involving the disequilibrium ratio, but the smaller contribution from the fractional saturation of the carrier (\( u/V_m \)) approaches 0 in the glucose and glucose plus ketones groups where [Glucose] >> [Glucose]. The reaction of UDP-Glc pyrophosphorylase was assumed to be in a state of near-equilibrium, and the elasticities were assigned as described above. The reaction of glycogen synthase was taken to be irreversible (Table IV).

Whenever \( \Gamma' \) was \( \leq 1 \) we assumed an equilibrium state, and where \( \epsilon_t \) and \( \epsilon_c \) approached negative and positive infinity, respectively, the numbers -100 or 100 were assigned to solve the equations using matrix algebra. In the glucose and ketones groups, \( \Gamma \) for the glucose transport step was far from equilibrium; intracellular glucose was low but several times higher than the \( K_c \) of hexokinase for glucose, so the use of glucose by hexokinase would be favored. The elasticity calculation does include a contribution for the reversibility in the term involving the disequilibrium ratio, but the smaller contribution from the fractional saturation of the carrier (\( u/V_m \)) approaches 0 in the glucose and glucose plus ketones groups where [Glucose] >> [Glucose]. The reaction of UDP-Glc pyrophosphorylase was assumed to be in a state of near-equilibrium, and the elasticities were assigned as described above. The reaction of glycogen synthase was taken to be irreversible (Table IV). The rate of glycogen synthesis was calculated \( u \) from the Michaelis-Menten equation (Equation 4) and substituted in Equation 6, and the derivative was taken.

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or insulin, respectively, but was not altered in the presence of both effectors (Table I). The increase in cardiac hydraulic work was primarily the result of an increase in systolic aortic pressure from 90.5 to 96.0 mm Hg. After the addition of ketones, insulin, or the combination, glycogen was synthesized at the rates of 0.67, 2.6, and 2.5 pmol of glucosyl units/min/ml of intracellular water, respectively. Significant differences from glucose-perfused hearts are indicated. When there were only two samples, S.E. pmol/min/ml of intracellular water. The numbers of observations in each group are as follows: glucose was combined with the measurement of 3H2O release at the P-glucoisomerase step, net glycolytic flux in hearts perfused with glucose alone was 5.04 pmol/min/ml of intracellular water in glucose-perfused hearts (Table I). As expected, net glycolytic flux decreased 3.0-fold on provision of the alternative substrate of ketone bodies and 2.3-fold on addition of insulin plus ketones; the addition of insulin alone caused no statistically significant change. The production of l-lactate at 10 cm H2O right atrial pressure accounted for only 0.5% of the glucose use in the glucose group and increased to 1, 7, and 0.7% in the ketones, insulin, and ketones plus insulin groups, respectively.

**Table I**

**Physiological and metabolic parameters in perfused working rat hearts**

Except for cardiac hydraulic work, the data are given as pmol/min/ml of intracellular water ± S.E. with the number of observations given in parentheses. Significant differences from glucose alone for lines 1 and 2 are indicated. Calculations in lines 3 and 4 are average values; standard error is based on the combination of error formula for the sum or difference of two figures, i.e., for \( z = x - y \), \( S_z = \sqrt{(S_x^2 + S_y^2)} \) where \( S_x \) is the standard error in \( x \), etc. Hydraulic work and \( {^3}\text{H}_{2}\text{O} \) production from [2-\( ^3\text{H} \)glucose were determined as described under “Experimental Procedures.”

| Metabolite | Glucose | Glucose + ketones | Glucose + insulin | Glucose + ketones + insulin |
|------------|---------|------------------|-------------------|---------------------------|
| Glycogen   | 21.4 ± 1.62 | 55.5 ± 2.95* | 112 ± 12.4* | 109 ± 3.31* |
| Glc-1-P    | 0.020 ± 0.004 | 0.035 ± 0.020 | 0.057 ± 0.012 | 0.198 ± 0.077* |
| Glc-1,6-P  | 0.007 ± 0.000 | 0.008 ± 0.002 | 0.013 ± 0.003* | 0.009 ± 0.001 |
| Glc-4-P    | 0.169 ± 0.010 | 0.612 ± 0.082* | 1.265 ± 0.020* | 1.626 ± 0.058* |
| Fru-6-P    | 1.91 ± 0.503 | 3.40 ± 1.12 | 11.5 ± 1.09* | 9.94 ± 0.712* |
| Fru-6-P (total) | 0.041 ± 0.005 | 0.150 ± 0.026* | 0.342 ± 0.003* | 0.321 ± 0.053* |
| DHAP       | 0.036 ± 0.005 | 0.111 ± 0.004 | 0.140 ± 0.047 | 0.593 ± 0.158 |
| GAP (x106) (calculated) | 1.62 ± 0.241 | 0.791 ± 0.179 | 2.50 ± 0.042* | 1.58 ± 0.221 |
| 1,3-P-glycerate (x106) (calculated) | 0.869 ± 0.081 | 3.32 ± 0.376* | 5.57 ± 1.10* | 2.51 ± 0.440* |
| 2,3-P-glycerate | 0.010 ± 0.004 | 0.008 ± 0.001 | 0.007 ± 0.002 | 0.010 ± 0.004 |
| 2-P-glycerate | 0.071 ± 0.004 | 0.063 ± 0.003 | 0.064 ± 0.018 | 0.042 ± 0.006* |
| P-enolpyruvate | 0.008 ± 0.002 | 0.009 ± 0.002 | 0.009 ± 0.002 | 0.002 ± 0.002 |
| Pyruvate    | 0.055 ± 0.004 | 0.036 ± 0.003* | 0.084 ± 0.004* | 0.080 ± 0.009 |
| L-lactate   | 0.683 ± 0.053 | 0.286 ± 0.064* | 0.737 ± 0.079 | 0.778 ± 0.247 |
| Citrate     | 1.432 ± 0.424 | 1.15 ± 0.076* | 1.68 ± 0.001* | 1.56 ± 0.210* |
| Isocitrate  | 0.018 ± 0.002 | 0.065 ± 0.004* | 0.051 ± 0.002* | 0.062 ± 0.009* |
| Fru-2,6-P6 (x106) | 11.3 ± 1.54 | 6.45 ± 1.72 | 8.39 ± 2.90 | 7.80 ± 0.51* |

* [Glycogen] was 35.3 ± 3.17 μmol of glucosyl units/ml of intracellular water at the end of the 15-min stabilization period.

b \( p < 0.05 \), Mann-Whitney U test.
the ketones plus insulin group. The activities of the enzymes of the glycolytic metabolism were analyzed as well as [cAMP], which can affect these enzymes through a cascade of protein kinases (69, 70). No significant change was found in cAMP (Table III) or in the percentage of phosphorylase a, the phosphorylated form. Total phosphorylase activity is given in Table V. The percentages in the a form were 5.6, 4.4, 5.4, and 5.0 in the glucose, ketones, insulin, and ketones plus insulin groups, respectively, suggesting that cytoplasmic free [Ca++] was not altered by the treatments given. Total glycerogen synthase was measured (Table V) and did not vary among the experimental groups. Despite the increases in phosphorylated hexoses, the immediate precursor of glycogen, [UDP-Glu], remained invariant; however, [UTP] decreased with addition of ketones and the combination, reflecting the increase in the 3-P-glycerate kinase reaction and the measured levels of 3-P-glycerate because the Vmax forward of 3-P-glycerate kinase is 15,000 μmol/min/ml of intracellular water, compared with 300 for that of the GAP dehydrogenase reaction and is 1 or 2 orders of magnitude greater than the other enzymes of glycolysis (Table V).

(3-P-glycerate) was 0.071 μmol/ml of intracellular water in hearts perfused with glucose alone and was unchanged on the addition of either ketones or insulin but decreased 1.7-fold on addition of ketones plus insulin. [2-P-glycerate] was decreased >3-fold in all groups as compared with glucose (Table II, Fig. 1).

In contrast to the up to 10-fold increases in metabolites preceding the P-fructokinase (EC 2.7.1.11) step, the concentrations of total Fru-1,6-P2 were decreased 4-fold by provision of the alternative substrate; ketone bodies increased 2-fold on addition of insulin, but remained unchanged by the combination (Table II, Fig. 1). Because of the large amount of Fru-1,6-P2 binding to aldolase (EC 4.1.2.13) (49), free [Fru-1,6-P2] was calculated from the measured DHAP and the K’ of the aldolase and triose-P isomerase reactions (Table IV). The free [Fru-1,6-P2] was calculated to be 0.68 mmol/l of intracellular water in hearts perfused with glucose alone and was altered by additions to the perfusate to the same degree as total [Fru-1,6-P2] (Table II, Fig. 1). The [Fru-2,6-P2], a positive effector of P-fructokinase (71, 72), was 11.3 mmol/ml of intracellular water in the glucose-perfused hearts and was 6.5 on addition of ketones, thus paralleling the changes in [Fru-1,6-P2]. In agreement with previous reports, insulin did not increase [Fru-2,6-P2] in the perfused hearts (73, 74).

DHAP was 0.036 mmol/ml of intracellular water in glucose-perfused hearts and increased 2.7-fold on addition of insulin, but it was unchanged on addition of ketones and the combination. [1,3-P2-glycerate], calculated from the K’ of the 3-P-glycerate kinase reaction and the measured levels of [3-P-glycerate] and cytoplasmic [ATP]/[ADP], was 0.87 μmol/ml of intracellular water, compared with 300 for the actual concentration. ICW, intracellular water; GI, glucose alone in the perfusate; GK, glucose plus ketone bodies; GI, glucose plus insulin; GK, glucose plus ketones and insulin; TCA cycle, tricarboxylic acid cycle.

**Fig. 1.** Metabolite concentrations (μmol/ml of intracellular water) in working perfused rat heart following the addition of 4 mm sodium L-β-hydroxybutyrate and 1 mm sodium acetocetate and/or insulin (100 nm). The differences from hearts perfused with glucose alone are shown as proportionate changes as described under "Experimental Procedures," with the concentrations in hearts perfused with glucose alone following the metabolite title; only glycogen is shown as the actual concentration. ICW, intracellular water; G, glucose alone in the perfusate; GK, glucose plus ketone bodies; GI, glucose plus insulin; GK, glucose plus ketones and insulin; TCA cycle, tricarboxylic acid cycle.

**Table III**

| Nucleotides, calculated pH, and free [Mg2+] in perfused working rat hearts |

|                  | Glucose | Glucose + ketones | Glucose + insulin | Glucose + ketones + insulin |
|------------------|---------|-------------------|-------------------|-----------------------------|
| pH               | 7.06 ± 0.01 | 7.05 ± 0.01 | 7.04 ± 0.02 | 7.02 ± 0.00* |
| Free (Mg2+)      | 1.23 ± 0.03 | 0.74 ± 0.01a | 0.73 ± 0.029 | 0.666 ± 0.082a |
| cAMP (x10³)      | 0.58 ± 0.031 | 0.74 ± 0.044 | 0.629 ± 1.04 | 0.856 ± 0.206 |
| UTP              | 0.19 ± 0.010 | 0.16 ± 0.021 | 0.179 ± 0.006 | 0.153 ± 0.005a |
| UDP              | 0.09 ± 0.002 | 0.02 ± 0.002a | 0.025 ± 0.001a | 0.022 ± 0.001a |
| UDP-Glc          | 0.099 ± 0.006 | 0.109 ± 0.015 | 0.096 ± 0.003 | 0.094 ± 0.001 |
| Phosphocreatine  | 0.74 ± 0.01 | 0.74 ± 0.044 | 0.629 ± 1.04 | 0.856 ± 0.206 |
| Creatine         | 0.19 ± 0.010 | 0.16 ± 0.021 | 0.179 ± 0.006 | 0.153 ± 0.005a |
| Cytoplasmic free [P] | 0.099 ± 0.006 | 0.109 ± 0.015 | 0.096 ± 0.003 | 0.094 ± 0.001 |
| Cytoplasmic [ATP]/[ADP] | 45.4 ± 2.55 | 168 ± 8.93a | 166 ± 9.50a | 179 ± 9.39a |

*p < 0.05, Mann-Whitney U test.
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The equilibrium constants ($K'$) have been corrected (62) where necessary for differences in tissue pH and free [Mg$^{2+}$] (Table II). The values of (products)/reactants) (i) are taken from Tables II and III. The reactions of UDP-Glc pyrophosphorylase, aldolase, triose-P isomerase, GAP dehydrogenase, 3-P-glycerate kinase, and pyruvate kinase are assumed to be at near-equilibrium, and $K'$ is given as 1. The values for $K'$ given at various pH and free [Mg$^{2+}$] are those for the sum of the species present. The reactions written are those of the most ionized species.

**TABLE IV**

Equilibrium constants of the reactions of glucose and glycogen metabolism compared with the ratio of [products]/[reactants] in perfused working rat hearts

| Reaction | Glucose | Glucose + ketones | Glucose + insulin | Glucose + ketones + insulin |
|----------|---------|------------------|------------------|---------------------------|
| Glycogen synthase (EC 2.4.1.11) | $K'$ | 37.7 | 29.8 | 29.5 | 28.5 |
| UDP-Glc$^2$ + Glc$_5$ → UDP$^2$ + Glc$_{4+1}$ + H$^+$ | $K'/$ | 0.343 | 0.193 | 0.261 | 0.294 |
| Phosphorylase (EC 2.4.1.1) | $K'$ | 0.719 | 0.723 | 0.727 | 0.716 |
| Glc$_4$ + P$^2$ → Glc$_{4+1}$ + Glc-1-P$^2$ | $K'/$ | 2.90 x 10$^{-3}$ | 4.47 x 10$^{-3}$ | 1.16 x 10$^{-2}$ | 3.08 x 10$^{-2}$ |
| UDP-Glc pyrophosphorylase (EC 2.7.7.9) | $K'$ | 4.36 | 3.47 | 3.34 | 3.46 |
| Glc-1-P$^2$ + UTP + H$^+$ → UDP-Glc$^2$ + HPP$^3$ | $K'/$ | 1 | 1 | 1 | 1 |
| Glucose transporter | | | | |
| Glc$_5$ → Glc$_4$ | $K'$ | 1 | 1 | 1 | 1 |
| Hexokinase (EC 2.7.1.1) | $K'$ | 2.09 x 10$^3$ | 2.42 x 10$^{1}$ | 2.49 x 10$^{2}$ | 2.43 x 10$^{2}$ |
| Glc + ATP → Glc-6-P$^2$ + ADP$^2$ + H$^+$ | $K'/$ | 1.94 x 10$^{-3}$ | 1.08 x 10$^{-4}$ | 6.64 x 10$^{-1}$ | 1.33 x 10$^{2}$ |
| P-glucomutase (EC 5.4.2.2) | $K'$ | 16.2 | 0.0617$^*$ | 0.0617$^*$ | 0.122 |
| Glc-1-P$^2$ → Glc-6-P$^2$ (glucose group) | $K'/$ | 8.40 | 0.057 | 0.045 | 0.198 |
| P-glucosidase (EC 5.3.1.9) | $K'$ | 0.30 | 0.30 | 0.30 | 0.30 |
| Glc-6-P$^2$ → Fru-6-P$^2$ | $K'/$ | 0.240 | 0.253 | 0.270 | 0.198 |
| P-fructokinase (EC 2.7.1.11) | $K'$ | 1.32 x 10$^{1}$ | 1.53 x 10$^{1}$ | 1.58 x 10$^{2}$ | 1.54 x 10$^{2}$ |
| Fru-6-P$^2$ + ATP → Fru-1,6-P$^2$ + ADP + H$^+$ | $K'/$ | 3.69 x 10$^{-3}$ | 6.09 x 10$^{-1}$ | 2.47 x 10$^{-3}$ | 1.08 x 10$^{-3}$ |
| Aldolase (EC 4.1.2.13) | $K'$ | 9.87 x 10$^{-5}$ | 9.82 x 10$^{-5}$ | 9.80 x 10$^{-5}$ | 9.83 x 10$^{-5}$ |
| Fru-1,6-P$^2$ → DHAP$^2$ + GAP$^2$ | $K'/$ | 1 | 1 | 1 | 1 |
| Triose-P isomerase (EC 5.3.1.1) | $K'$ | 0.046 | 0.046 | 0.046 | 0.046 |
| DHAP$^2$ → GAP$^2$ | $K'/$ | 0.046 | 0.046 | 0.046 | 0.046 |
| GAP dehydrogenase (EC 1.2.1.12) | $K'$ | 3.04 x 10$^{-1}$ | 3.82 x 10$^{-1}$ | 6.00 x 10$^{-1}$ | 5.50 x 10$^{-1}$ |
| GAP$^2$ + P$^2$ + NAD$^+$ → 1,3-P$_2$-glycerate$^+$ + NADH + H$^+$ | $K'/$ | 6.04 x 10$^{-3}$ | 3.82 x 10$^{-1}$ | 6.00 x 10$^{-1}$ | 5.50 x 10$^{-1}$ |
| 3-P-glycerate kinase (EC 2.7.2.3) | $K'$ | 3767 | 3094 | 3075 | 2942 |
| 1,3-P$_2$-glycerate$^+$ + ADP$^2$ → 3-P-glycerate$^2$ + ATP | $K'/$ | 3767 | 3094 | 3075 | 2942 |
| 3-P-glycerate mutase (EC 5.4.2.1) | $K'$ | 0.11 | 0.11 | 0.11 | 0.11 |
| 3-P-glycerate$^2$ → 2-P-glycerate$^3$ | $K'/$ | 0.195 | 0.071 | 0.047 | 0.076 |
| Enolase (EC 4.2.1.11) | $K'$ | 4.0 | 4.0 | 4.0 | 4.0 |
| 2-P-glycerate$^2$ → P-enolpyruvate$^3$ | $K'/$ | 1.47 | 2.30 | >4.0 | 1.39 |
| Pyruvate kinase (EC 2.7.1.40) | $K'$ | 1.20 x 10$^{1}$ | 1.10 x 10$^{4}$ | 1.07 x 10$^{4}$ | 1.09 x 10$^{4}$ |
| P-enolpyruvate$^3$ + ADP + H$^+$ → Pyruvate$^6$ + ATP | $K'/$ | 1.91 x 10$^{-1}$ | 5.82 x 10$^{-1}$ | 1.18 x 10$^{-1}$ | 2.44 x 10$^{-1}$ |
| Lactate dehydrogenase (EC 1.1.28) | $K'$ | 7.85 x 10$^{1}$ | 8.22 x 10$^{4}$ | 8.03 x 10$^{4}$ | 8.60 x 10$^{4}$ |
| Pyruvate$^6$ + NAD$^+$ + H$^+$ → l-Lactate$^-$ + NAD$^+$ | $K'/$ | 7.85 x 10$^{1}$ | 8.22 x 10$^{4}$ | 8.03 x 10$^{4}$ | 8.60 x 10$^{4}$ |

* R. N. Goldberg, personal communication. Calculated from $\Delta G^\circ$ values for the hydrolysis of sucrose (41) and maltose (42) and synthesis of sucrose (34), (44), (35), (46), (47), (48), (49), (50), (51), (52), (53), (54).

p < 0.05, Mann-Whitney U test.

...and after perfusion with insulin it was not detectable by our methods. There was, however, no change in [2,3-P$_2$-glycerate], indicating that the decrease in [2-P-glycerate] did not result from lack of the cofactor for 3-P-glycerate mutase. [P-enolpyruvate] was significantly decreased in the presence of ketones with insulin but was unchanged in the other experimental
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**TABLE V**

Kinetic parameters of the enzymes of glucose and glycogen metabolism

The kinetics of the glycolytic enzymes were measured in 10 mM Pi, 20 mM imidazole buffer, pH 7.2, 150 mM KCl, and 5 mM total magnesium at 38 °C. Activity is expressed as μmol/min/ml of intracellular water ± S.E., and *K*ₐₐ values are expressed as mM. The number of observations is 3. *V*ₘₐₓ and *K*ₐₐ denote the velocity and *K*, in the direction of l-lactate formation (forward); *V*ₘₐₓ and *K*ₐₐ denote the same parameters in the opposite direction (reverse).

| Enzyme                          | *V*ₘₐₓ  | *K*ₐₐ  | *V*ₘₐₓ  | *K*ₐₐ  | *K*ₐₐ nucleotide or Pi |
|--------------------------------|---------|---------|---------|---------|----------------------|
| Glycogen synthase              | 8.81 ± 1.65 | 0.08* | 8.81 ± 1.65 | 1.42* |                     |
| Phosphorylase                   | 46.9 ± 2.56 | 0.1* |                     | 3350 | 5*                   |
| P-glucomutase                  | 116 ± 16.6 | 0.045 | 67.2 ± 3.15 | 0.67 |                     |
| Glucose transport              | 4        | 2      |                     |       |                      |
| 3-O-Methylglucose              | 0.14–1   | 7–10   | 1–0.01 | 7      |                      |
| Without insulin                | 13.2     | 6      | 6.6     | 3      |                      |
| With insulin                   | 33 ± 1.35 | 0.072 | 0.00637* | 0.042* | 0.236 (ATP)          |
| Hexokinase                     | ND       |        |         |        |                      |
| Glucose-6-phosphatase          | 5.70     |        |         |        |                      |
| Glc-6-P dehydrogenase          | 604 ± 47.8 | 0.425 | 576 ± 12.5 | 0.175 |                     |
| P-glucoisomerase               | 79.7 ± 8.26 | 0.224 |         | 0.127 |                     |
| P-fructokinase                 | 59.5 ± 2.50 | 0.038 |         |        |                      |
| Aldolase                       | 356 ± 32.5 | 1.53  |         |        |                      |
| Triose-P isomerase             | 321 ± 28.7 | 0.042 |         |        |                      |
| GAP dehydrogenase              | 15,060 ± 175 | 0.021 | 959 ± 169 | 0.51  |                     |
| 3-P-glycerate kinase           | 674 ± 77.8 | 0.145 | 2860 ± 101 | 0.139 |                     |
| 3-P-glycerate mutase           | 111 ± 6.22 | 0.045 | 120 ± 7.40 | 0.089 |                     |
| Enolase                        | 556 ± 18.6 | 0.11  | 0.63*  | 10*   | 0.268 (ADP)          |
| Pyruvate kinase                | 1436 ± 36.6 | 0.125 |         |        | 0.001 (NADH)³       |
| Lactate dehydrogenase          |         |        |         |        |                      |
| *a* *K*ₐₐ for the I and D forms in the presence of G6-P (55).  
*¹* *V*ₘₐₓ was calculated by Haldane’s relationship.  
*²* Ref. 56.  
*³* Ref. 4, µmol/g wet weight.  
*⁴* Calculated from Ref. 57.  
*⁵* Not detected.  
*⁶* *Kₐₐ* for NAD⁺ was increased by increasing [Pi], and *Kₐₐ* for Pi was increased by increasing [GAP]. The value for NAD⁺ reported here is in the presence of 5 mM Pi, and that for Pi is in the presence of 0.036 mM GAP.  
*⁷* Ref. 53.  
*⁸* *Kₐₐ* for NADH was increased in the presence of increased [pyruvate]. The value reported here was determined in the presence of 0.022 mM pyruvate.

Because the binding of GAP to aldolase distorts the tissue ratios, the *I/K* values for the reactions catalyzed by aldolase and triose-P isomerase were assumed to be at near-equilibrium (49) and, therefore, were assigned a value of 1. [GAP] was calculated from the measured value of [DHAP] and the *K* of the triose-P isomerase reaction. For similar reasons and because of the instability of 1,3-P₂-glycerate, the tissue levels of these metabolites were not measured but were calculated from the components of the 3-P-glycerate kinase reaction. The values of *I/K* for the GAP dehydrogenase and the 3-P-glycerate kinase reactions were assigned a value of 1 (51, 75). *I/K* for the components of the 3-P-glycerate mutase reaction was >1 during perfusion with glucose alone and decreased to 0.65 on addition of ketones or 0.59 with ketones plus insulin, but it decreased to 0.42 on perfusion with insulin alone. *I/K* for the enolase reaction was 0.37 on perfusion with glucose alone and 0.35 in the presence of ketones plus insulin, but it increased to 0.58 on addition of ketones and >1 on perfusion with insulin. The apparent changes in the flux control coefficient at the enolase and P-glycerate mutase steps were an unexpected outcome of this type of analysis. The changes did not correlate with changes in the measured amounts of the cofactor of the mutase, 2,3-P₂-glycerate (Table II). In contrast to the findings with the substrates of the hexokinase and P-fructokinase reactions where *I/K* was <10⁻⁶ or the 3-P-glycerate kinase reaction where *I/K* was 1, the *I/K* values for the substrates of the pyruvate kinase reaction were a factor of 100 from equilibrium. *I/K* for the pyruvate kinase reaction was 0.016 during perfusion with glucose alone and in-

Groups despite the decrease in the precursor, [2-P-glycerate]. [Pruyvate] was 0.055 µmol/ml of intracellular water in hearts perfused with glucose alone, decreased 1.5-fold on addition of ketones, increased 1.5-fold on addition of insulin, and was unchanged on addition of ketones plus insulin. [l-Lactate] was 0.68 µmol/ml of intracellular water in hearts perfused with glucose alone and decreased 2.3-fold on the addition of ketones but was unchanged on the addition of insulin or insulin plus ketones. The l-lactate efflux increased 1.7-, 1.3-, and 2.2-fold in the presence of ketones, insulin, or the combination, respectively.

The values of the equilibrium constants (*K*) of the reactions catalyzed by the enzymes of glucose metabolism, corrected for changes in pH and free [Mg²⁺] when appropriate, were compared with the values of the [products]/[reactants] of the measured concentrations in heart tissue (Table IV). The *I/K* for the glucose transport reaction was 0.19 during perfusion with glucose alone, 0.34 after addition of ketones, and about 1 on addition of insulin and the combination, showing that insulin equilibrated [Glc] and [Glc]. The reactions catalyzed by hexokinase and P-fructokinase had *I/K* ranging from 10⁻⁸ to 10⁻⁵, confirming previous reports in other tissues that these steps were far from equilibrium (45, 76). For the reactions of glycogen synthase and phosphorylase, the *I/K* ranged between 10⁻² and 10⁻³. The *I/K* of the reaction catalyzed by P-glucosomerase ranged from 0.90 to 0.66 despite the 10-fold increase in [Glc-6-P], showing that this step is at near-equilibrium under all conditions tested here.
in the presence of glucose only, the flux control coefficient of fluxes used in the calculation of flux control coefficients in order to calculate the sensitivity and error analysis. Values are given ± S.E.; the coefficient of variation (S.D. mean × 100) in percent is given in parentheses.

| Glucose | Glucose + ketones | Glucose + insulin | Glucose + ketones + insulin |
|---------|-----------------|-----------------|-----------------------------|
| C\textunderscore\text{Glpd} | -0.237          | -0.516          | -100                        |
| C\textunderscore\text{Glc} | 0.159           | 0.248           | 0.164                       |
| C\textunderscore\text{Gip} | 0.128           | -0.232          | -0.159                      |
| C\textunderscore\text{Glpd} | -1.22           | 12.9            | 3.18                        |
| C\textunderscore\text{Glpd} | 1.81            | -12.5           | -2.94                       |
| C\textunderscore\text{Glpd} | 4.75            | 5.91            | 9.39                        |
| C\textunderscore\text{Glpd} | NA             | 100             | 100                         |
| C\textunderscore\text{Glpd} | NA             | NA              | NA                          |
| C\textunderscore\text{Glpd} | -0.011          | NA              | NA                          |
| C\textunderscore\text{Glpd} | 0.396 ± 0.34 (9.2) | 0.314 ± 0.030 (9.6) | 0.002 ± 0.0002 (10) |
| C\textunderscore\text{Glpd} | 0.590 ± 0.033 (5.6) | 0.655 ± 0.029 (4.4) | 0.972 ± 0.003 (3.1) |
| C\textunderscore\text{Glpd} | 0.97           | 0.001           | -0.002                      |
| C\textunderscore\text{Glpd} | 0.016 ± 0.002 (13) | 0.024 ± 0.003 (13) | 0.016 ± 0.003 (19) |
| C\textunderscore\text{Glpd} | NA             | <0.001          | <0.001                      |
| C\textunderscore\text{Glpd} | NA             | 0.009 ± 0.002 (22) | 0.009 ± 0.002 (22) |
| C\textunderscore\text{Glpd} | NA             | NA              | NA                          |
| C\textunderscore\text{Glpd} | -0.001 ± 0.0001 (10) | NA              | NA                          |

2. Linear pathway from 3-P-glycerate kinase to pyruvate kinase

| C\textunderscore\text{Glpd} | C\textunderscore\text{Glpd} | C\textunderscore\text{Glpd} | C\textunderscore\text{Glpd} |
|-----------------|-----------------|-----------------|-----------------|
| 100             | -100            | -100            | -100            |
| 2.50            | 1.39            | 3.06            |                 |
| 1.43            | 2.27            | 1.47            |                 |
| -0.687          | -1.45           | -1.24           |                 |
| 0.859           | 0.929           | 0.981           | 1.23            |
| 0.008 ± 0.002 (25) | 0.008 ± 0.001 (13) | 0.008 ± 0.001 (13) | 0.009 ± 0.002 (22) |
| 0.008 ± 0.001 (13) | 0.326 ± 0.036 (11) | 0.575 ± 0.048 (8.3) | 0.306 ± 0.31 (11) |
| 0.547 ± 0.034 (6.2) | 0.260 ± 0.019 (7.3) | 0.084 ± 0.004 (10) | 0.466 ± 0.025 (5.4) |
| 0.438 ± 0.035 (8.0) | 0.406 ± 0.039 (9.6) | 0.412 ± 0.048 (12) | 0.219 ± 0.029 (11) |

*Scheme 1.

†NA, not applicable.

‡Scheme 2.

creased to 0.053 on addition of ketones, 0.11 on addition of insulin, and 0.22 on addition of the combination. There was no correlation between the shifts for the pyruvate kinase reaction and the [%Fru-1,6-P₂] or [%Fru-2,6-P₂]. Using bottom-up analysis of the upper portion of glucose metabolism (Scheme 1), from extracellular glucose to Fructose-6-P and glycogen (Table VI, part 1), the flux control coefficient of glucose transporter (C\textunderscore\text{Glpd}) was 0.40, of hexokinase (C\textunderscore\text{Glpd}) was 0.59, of P-glucoisomerase (C\textunderscore\text{Glpd}) was 0.02, and of phosphorylase (C\textunderscore\text{Glpd}) was -0.001 during perfusion with glucose alone. The activity of glucose-6-phosphatase was not considered since we were unable to detect it in vivo, although this activity has been inferred from NMR data. The addition of ketones slightly increased control at the hexokinase and P-glucoisomerase steps; some control appeared at the glycogen synthase step (C\textunderscore\text{Glpd}). Insulin essentially abolished control at the glucose transporter; C\textunderscore\text{Glpd} = 0.002, C\textunderscore\text{Glpd} = 0.97, C\textunderscore\text{Glpd} = 0.02, and C\textunderscore\text{Glpd} = 0.01. In the presence of insulin and ketones, control was shared by hexokinase, P-glucoisomerase, and glycogen synthase; C\textunderscore\text{Glpd} = 0.86, C\textunderscore\text{Glpd} = 0.07, and C\textunderscore\text{Glpd} = 0.07. In the terminal glycolytic portion of bottom-up analysis (Scheme 2), from 1,3-P₂-glycerate to pyruvate (Scheme VI, part 2); in the presence of glucose only, the flux control coefficient of enolase (C\textunderscore\text{Glpd}) was 0.55 and of pyruvate kinase (C\textunderscore\text{Glpd}) was 0.44, whereas the flux coefficients of 3-P-glycerate kinase (C\textunderscore\text{Glpd}) and 3-P-glycerate mutase (C\textunderscore\text{Glpd}) were <0.01. After addition of ketones, insulin, or both, C\textunderscore\text{Glpd} = 0.33, 0.58, and 0.31, respectively. C\textunderscore\text{Glpd} was decreased to 0.26 after addition of ketones and to 0.47 in the presence of ketones plus insulin, but it was reduced to nearly 0 in the presence of insulin and absence of ketones. C\textunderscore\text{Glpd} was not changed in the presence of ketones or insulin, but it was decreased to 0.22 in the presence of the combination.

Using top-down analysis with Glc-6-P as the intermediate between blocks (Scheme 4), C\textunderscore\text{Glpd} comprising the block of glucose transport and phosphorylation step was 0.58, C\textunderscore\text{Glpd} of the block comprising glycolysis was 0.17, and C\textunderscore\text{Glpd} of the block comprising glycolysis was 0.26. This type of analysis confirmed that glucose entry and phosphorylation dominated the control of glucose metabolism, with glycolysis exerting about a quarter and glycogen synthesis 17% of the control of the total rate (Table VII, part 1). Top-down analysis was performed with Glc-6-P as the intermediate between blocks composed of (Scheme 4) C\textunderscore\text{Glpd}, the glucose transporter, and C\textunderscore\text{Glpd}, all of the other reactions of glucose utilization. In the presence of ketones, the control distribution was similarly shared between the two blocks; C\textunderscore\text{Glpd} = 0.55 and C\textunderscore\text{Glpd} = 0.45 (no significant difference). This contrasts with the states involving addition of insulin where C\textunderscore\text{Glpd} de-
creased to nearly zero, while the control exerted by the combined pathways of glucose utilization increased to 0.99 (Table VII, part 2). Although these top-down results are approximate, they offer corroboration (via an independent method of analyzing the results) of the conclusion from the bottom-up analysis that in the absence of insulin the transporter shares some of the control but that in the presence of insulin the control shifts to glucose utilization.

**DISCUSSION**

In a metabolic pathway in the steady state,

\[
\begin{align*}
\text{Glc} & \xrightarrow{v_1} \text{Gluc_4} \xrightarrow{HK} \text{Glc_6P} \quad \text{PGM} \quad \text{GIP} \\
\end{align*}
\]

the flux (\(J\)) through the pathway is equal to the net rate (\(v\)) for any single enzyme step, all of which are equal. Since \(v_1 = v_2 = v_3 = J\), it is true, but misleading, to say that the flux through the pathway is equal to the “slowest” or “pacemaker” enzyme since the flux through all of the steps is equal in the steady state. By assigning flux control coefficients to each step or to blocks of steps and by setting \(\sum C_i = 1\) (the summation theorem, Equation 9), metabolic control analysis attempts to define formally the proportion of a change in the flux that is caused by changes either in the reactants or in the kinetic properties of each particular step in an arbitrarily defined pathway. This analysis shows that control of metabolic flux can be distributed between several enzymatic steps and that the site and degree of control may vary. Thus control of the flux of glucose utilization varies with the nutrient presented or the receptor stimulated. This has implications for both genetic and pharmacological therapy in that alteration in the degree of flux control at one step will result in the development of flux control at other steps. It is therefore necessary to understand the metabolic system as a whole and not simply focus narrowly on one or another enzyme step within the pathway.

Flux control coefficients may be derived from the elasticity of each enzyme step using the connectivity theorem (Equation 10, \(\sum C_i^J = C_i^{\text{in}} = 0\)). Elasticities of small magnitude are critical; large elasticities have relatively little influence on the flux control coefficient. If two adjacent enzymes have a large and small elasticity, respectively, for a common intermediate, the enzyme with the small elasticity will have the larger flux control coefficient. A negative value for elasticity means an inhibitory effect (elasticities embody the kinetic effects). For example, with [Glc] as the common intermediate, the glucose transporter and hexokinase are adjacent enzymes. Equation 10 can be described as \(C_{\text{Gluc}_4}^{\text{Glc}_6P} + C_{\text{HK}}^{\text{Gluc}_4} = 0\). The value of \(e_{\text{Glc}_6P}^{\text{Glc}_4}\) was -0.24, and \(e_{\text{HK}}^{\text{Gluc}_4}\) was 0.16 in the glucose group; \(C_{\text{Gluc}_4}^{\text{Glc}_6P}\) was 0.40, and \(C_{\text{HK}}^{\text{Gluc}_4}\) was 0.59.

As long as the flux control coefficient can be obtained from the elasticities, the change in flux is a result of changes in either the inherent kinetic properties of the enzyme or in the ratio of [products] to [reactants] for that enzyme. The explanation for this relationship may be seen from the derivation of elasticities (Equation 8), which are a function of \(K\) for the reaction, the ratio of [products] to [reactants] actually achieved under the condition studied (here called \(\Gamma\)), and \(V_{\text{max}}\) in the forward and backward direction for each enzyme. In the case of the enzymes of glucose utilization in the perfused heart, the elasticities for each step fall into three classes depending upon how close they come to catalyzing equilibrium between their products and reactants under the conditions studied.

When \(1/K\) of an enzyme-catalyzed reaction is >0.5 (close to equilibrium), the elasticity depends primarily on the value of \(1/K\), and as \(1/K\) increases, the value of \(\varepsilon\) increases in a manner resembling an exponential curve. In perfused hearts, this situation pertains to the P-glucosomerase and P-glucosamine reactions (Table IV). Analytically, it is difficult to distinguish between 0.5 and 1 of \(1/K\); however, in this range the elasticity will change by orders of magnitude and will, in turn, affect the calculation of the flux control coefficients. In some instances, this relationship assigns flux control coefficients that appear to vary disproportionately with the observed changes in [S]. For example, in the cases of enolase and P-glycerate mutase, small changes in [2-P-glycerate] and [P-enolpyruvate] resulted in the shift of control from one enzyme to the other. The low concentrations lend a degree of uncertainty to the measurement of these metabolites, which in some conditions were below the range of accurate detection.

When \(1/K\) is >0.01 but <0.5, elasticity is a function both of the ratio of [products] to [reactants] and of the kinetic constants for the enzyme. An example of such a reaction is the insulin-sensitive glucose transporter, Glut4 (7). During perfusion with glucose alone, \(1/K\) was 0.19, and thus Glut4 would be sensitive not only to changes in the ratio of [products] to [reactants] but also to changes in the kinetic constants of the enzyme. Both mechanisms were demonstrated here. By the first mechanism, addition of ketones decreased the demand for glucose and resulted in an increase in [Glc] from 1.9 to 3.5 \(\mu\)mol/ml of intracellular water (Table II). The elevation in [product] increased \(1/K\) from 0.19 to 0.34, altering \(e_{\text{Glc}_6P}\) from -0.24 to -0.52 (Table VI). Second, addition of insulin changed the kinetic constants of the glucose transporter by increasing \(V_{\text{max}}\) 10-fold (Table V), which increased [Glc] to near 10 \(\mu\)mol/ml of intracellular water (Table II), making \(1/K\) near 1; and thus \(e_{\text{Gluc}_4}\) was increased to negative infinity (-100 was arbitrarily assigned) with a consequent decrease in \(C_{\text{Gluc}_4}^{\text{Glc}_6P}\) to nearby zero. The very low flux control coefficient of the glucose transporter in the presence of insulin means that under conditions of saturating doses of insulin, glucose transport no longer plays a significant role in the control of glucose utilization.

In the case of the hexokinase reaction, product inhibition by Glc-6-P significantly decreases the flux control coefficient of this step below what it might be were such inhibition not present. The significance of this effect has been pointed out by other workers (77).

In those enzymes that catalyze reactions that are far from equilibrium so that \(1/K\) is <0.01, such as hexokinase, P-fructokinase, and (in most cases) glycogen synthase and phosphorylase, the elasticity is totally insensitive to changes in \(1/K\) and responds only to changes in kinetic constants.

The flux control coefficient is also affected by the ratio of the fluxes in the branches, which is equivalent to the ratio of the flux control coefficients (the branch point theorem, Equation 11). Despite the small elasticities of glycogen synthase and phosphorylase, \(C_{\text{Glyc}4}\) in the presence of ketones, insulin, and ketones plus insulin and \(C_{\text{Fasg}}\) in the presence of glucose were small; this is in part a consequence of the flux ratios. \(C_{\text{Glyc}4}\) and \(C_{\text{Fasg}}\) were also affected by the neighboring UDP-Glc pyrophosphorylase and P-glucosamine reactions in the same elasticities.

Finally, the flux control coefficients were obtained by using matrix algebra (Equations 12, 15, 17, 20, 22, and 24); the proportion of control will be affected by each elasticity, the ratio of the fluxes, and the other flux control coefficients in the system.

The conversion from glycogen phosphorylase to glycogen synthesis was achieved in two ways. First, the requirement for glucose was decreased by providing ketone bodies as an alternative substrate for oxidative phosphorylation, permitting the accumulation of glucose and Glc-6-P. Second, in the presence of
Ketone Bodies and Insulin Action on Glucose Utilization

Table VII
Flux control coefficients determined by top-down analysis

| Block                              | Control        | Addition        | C      |
|------------------------------------|----------------|-----------------|--------|
| 1. Glucose transport and phosphorylation | Glucose        | Ketones         | $\varepsilon_{Glucose}$ | $-0.51 \pm 0.26$ | $C'$ | 0.58 $\pm$ 0.17 (29) |
| 2. Glycogen synthesis              | Ketones        | Insulin         | $\varepsilon_{Ketones}$ | $1.34 \pm 0.47$ | $C'$ | 0.17 $\pm$ 0.07 (42) |
| 3. Glycolysis                      | Ketones        | Insulin         | $\varepsilon_{Ketones}$ | $0.26 \pm 0.48$ | $C'$ | 0.26 $\pm$ 0.11 (42) |

2. Glc, as intermediate

| Block 2 | Control        | Change Value   | Value   |
|---------|----------------|----------------|---------|
| $\varepsilon_{Glucose}$ | Glycogen synthesis and glycolysis | Glucose | Insulin | 0.18 $\pm$ 0.10 |
| $\varepsilon_{Ketones}$ | Ketones | Insulin | 0.63 $\pm$ 0.35 |

| Flux control coefficients indicate the distribution within that selected pathway only; they would all be scaled down if the whole of glucose metabolism was considered. Viewed from the perspective of the processes of glucose utilization, and using top-down analysis, the proportion of control of the block of glucose transporter and phosphorylation, glycogen metabolism, and glycolysis was determined with [Glc-6-P] assigned as the intermediate metabolite. The flux control coefficient of glucose transport and phosphorylation was 0.58, that of glycogen synthesis was 0.17, and all of glycolysis was 0.26, which is an approximate average over the states considered (Table VII, part 1). It is clear from this study that, considering glucose metabolism as a whole, the activity of P-fructokinase is not the pacemaker, since only 0.26 of the control of glucose utilization is found in all of the steps below P-glucoisomerase. That is not to say that P-fructokinase does not have an elegant system of controls that involve an increasing number of previously unknown effectors formed by enzymes undergoing covalent modification (71, 72, 79). However, the flux through the branches of Fru-6-P and GAP into the nonoxidative portion of the hexose monophosphate pathway (80) and purine synthesis and salvage (81) and the flux through the branch of DHAP to $\alpha$-glycerophosphate and the $\alpha$-glycerophosphate shuttle (82) (Fig. 1) were not measured. The absence of precise flux measurements through P-fructokinase and the multiple branch points above and below the P-fructokinase reaction precludes the possibility of performing bottom-up analysis at the P-fructokinase step in this study.

In addition to preventing misstatement, the application of this formal approach to an entire pathway provides new insights that may further our understanding of control. An example is the finding that $K'/K$ values for phosphorolysine, pyruvate kinase, and P-glycerate mutase range from 0.01 to 0.5 and thus have characteristics more in common with the glucose transporter than with enzymes of the type such as hexokinase, P-fructokinase, and glycogen synthase on the one hand (where $K'/K < 0.01$) or with P-glucoisomerase on the other (where $K'/K > 0.5$). Enzymes that have intermediate values of $K'/K$ might be expected to change the degree to which they may affect flux through the lower portion of glycolysis in response to changes in substrate and product concentrations and to changes in their fundamental kinetic parameters.

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