**Supplementary Text**

**for “The Dynamic Side of the Warburg Effect: Glycolytic Intermediates as Buffer for Fluctuating Glucose and O2 Supply in Tumor Cells” by JHGM van Beek**

Short Description of the Computational Model

Chance and Hess explained their kinetic data on Ehrlich ascites tumor cells (EATC) with the first computational model of a multi-enzyme system ever published (*1, 2*), incorporating biochemical assumptions which are now considered false. A new computational model is developed here which simultaneously describes Warburg’s measurements on steady EATC metabolism (*3*) and kinetic data during the first minutes by Chance (*1, 4, 5*), Coe (*6, 7*) and others (*8, 9*). The approach is similar to recent modeling of yeast glycolysis to show trade-offs between robustness and efficiency under non-limiting glucose supply (*10*). The new model gives a simplified representation of the EATC metabolic system with the aim to reproduce a broad range of metabolic responses of EATC.

Blood flow in tumor tissue is often low and fluctuating, resulting in cycling hypoxia (*11-13*) and glucose shortages. The present model therefore includes the dependence of glycolysis and mitochondrial ATP production on glucose and oxygen concentrations. Glycolysis is represented by a head section which produces phosphorylated glycolytic intermediates (PGI), for the major part fructose 1,6-bisphosphate (FBP), from glucose and ATP. In the glycolytic tail section, downstream from FBP, ADP is phosphorylated to ATP (Figure 1). Inhibition of the head section by PGI constitutes an important regulatory feedback loop. Strong negative feedback by glucose 6-phosphate on hexokinase in tumor cells (*14*) and by FBP on the head section of glycolysis in EATC (*15*) is well known. This inhibition shows >10 s time delay (*15, 16*).

Model Equations

Kinetic Rate Equations

Jhead, the flux in the head section of glycolysis, where glucose is twice phosphorylated, is quantified as the amount of six-carbon units taken up per liter intracellular water per second (µM/s). The reactants of the head section are glucose and 2 ATP molecules; the products of the head section are fructose 1,6-bisphosphate (FBP) and 2 ADP. The rate equation resembles the equation for hexokinase (*17, 18*), the enzyme which dominates flux control over glycosis in cancer cells (*14*). There is strong feedback inhibition of hexokinase by glucose 6-phosphate (*14*). Feedback inhibition of this enzyme by FBP was also described specifically for EATC (*15, 19*). The third enzyme in the head section, phosphofructokinase (PFK), is inhibited in EATC by phosphoenolpyruvate (PEP), another PGI species (*20*). These inhibition mechanisms of the head section by downstream metabolites are implemented by modulation of the fraction of the head section which is active, Factive. This is regulated by FBP, representing the PGI pool, via forward and backward rate constants, see Eq. 22. The rate equation therefore describes the effects of glucose and ATP concentrations and feedback inhibition from the PGI pool:

 (1)

Variables and parameters in all equations are described in Supplementary Tables 1-3.

In the tail section of glycolysis FBP, inorganic phosphate (Pi), nicotinamide adenine dinucleotide (NAD) and ADP are used; pyruvate, ATP and NADH are produced with flux Jtail. The flux unit here is amount of three-carbon units per liter intracellular water per second (µM/s). The rate depends on the concentrations of the reactants FBP, NAD and ADP and is decreased by the product NADH:

 (2)

Mitochondrial ATP synthesis by oxidative phosphorylation depends on ADP, Pi and O2 (*21-23*):

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 (3)

The sensitivity of respiration in EATC to low oxygen concentrations has been determined by Froese (*22*). Although Pi may play a regulatory role in EATC metabolism if external phosphate concentrations are low, changes in metabolism are saturated at high external phosphate concentrations and regulation is shifted to ADP (*2, 5, 24, 25*). The experiments simulated here were conducted at high extracellular inorganic phosphate concentrations (*6, 26*), associated with high intracellular phosphate concentrations (*27, 28*). Measured intracellular Pi levels are well above the Km’s of oxidative phosphorylation (*2, 23*) and glyceraldehyde 3-phosphate dehydrogenase (*25*). Metabolic regulation is therefore saturated with phosphate at these high phosphate concentrations in EATC (*24*). Variation of Pi and its effect on metabolic regulation can therefore be neglected, avoiding unnecessary complexity of the model: intracellular inorganic phosphate concentration was therefore fixed at 6000 μM in the model, close to saturating for regulation of oxidative phosphorylation and glycolysis.

Lactate dehydrogenase catalyzes a reversible reaction, pyruvate + NADH ⇌ lactate + NAD, depending on reactant and product concentrations. The rate equation is (*29*):

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 (4)

In order to explain the time course of the measured metabolites carrying phosphate groups following glucose addition *in vitro*, a strong decrease of ATP hydrolysis, Jhyd,ATP, proves essential. This decrease mainly affects processes such as macromolecular synthesis and degradation, ion pumping, etc. (*30*). The decline in Jhyd,ATP is clearly revealed by calculating the mass balance of phosphate metabolites directly from the experimental data (see Figure 2). This decline is assumed to result from decreased availability of energy-delivering adenine nucleotides. The highest correlation of falling ATP hydrolysis is not with ATP or ATP/ADP, but with the sum AdN = ATP + ADP of double and triple phosphorylated adenine nucleotides. The decrease in AdN in turn corresponds with the sum of accumulated breakdown products of ATP and ADP such as AMP, inosine, adenosine and hypoxanthine (*31, 32*). ATPinitialandADPinitialare the initial ATP and ADP concentrations in the fully energized state before the intervention. AdNinitial = ATPinitial+ADPinitial. In the model, Jhyd,ATP is therefore declining linearly with the adenine nucleotide concentration:

 (5)

In the *in vitro* experiments (Figure 2), AdN reaches a minimum value, AdNcutoff , which is used as a threshold in further simulations (see below). Different cellular processes have a quite different sensitivity to lowering of energy status, ATP and/or AdN levels. Protein synthesis is very sensitive to declining ATP content with a 20% decline causing ~60% decline in ATP hydrolysis (*33*), similar as the energy dependence of ATP hydrolysis determined in the *in vitro* experiments of Figure 2. Indeed, protein synthesis is the highest ATP-consuming process in EATC (*30*). In contrast, the sensitivity of protein degradation is relatively low with a 70% fall in ATP content causing a ≤ 30% decrease in protein breakdown (*33*). The sensitivity of ion pumps to ATP is between these extremes (*34*).

When AdN falls below AdNcutoff in simulations of low nutrient and O2 supply conditions in tissue, Vhyd is given by a curve continuous with Eq. 5:

 (6)

The linear term with khyd,AdN,low = 0.0098704 s-1 defines a sensitivity which is similar as found for protein synthesis at low energy status below AdN­cutoff (*33*). The linear relation at low AdN is joined to the linear relation at high AdN in Eq. 5 via the power term in Eq. 6 which has a high value for γ so that is has a negligible effect on the linear relation at low AdN. The coefficient for the power term, C­power, and the power γ are determined such that Eqs. 5 and 6 together form a smooth function which is continuous and differentiable at AdN = AdNcutoff.

For the simulations in Figures 3 and 4 and Supplementary Figure 3 a high khyd,AdN was chosen for the upper part of the curve in Eq. 5 (Supplementary Table 3), reflecting a strong decrease of Jhyd,ATP if AdN falls. This is the value determined in the *in vitro* experiment of Figure 2. Eqs. 5 and 6 together define a “hockeystick” curve similar to the energy dependence of protein synthesis in Fig 8 of Gronostajski et al. (*33*), with slope increasing with AdN. By using this strong reduction of ATP hydrolysis, the energy homeostatic mechanism of reduction of ATP hydrolysis is active in these tissue simulations. Eqs. 5 and 6 can jointly define convex or concave curves similar as found experimentallyfor various ATP consuming processes (*33*). For the simulations in Supplementary Figure 4 a much lower sensitivity of ATP hydrolysis to energy status was chosen, resembling the energy dependence of protein hydrolysis (see details below).

Eq. 7 represents ADP breakdown to AMP when the energy status decreases (*31, 32*). AMP is broken down further to adenosine, inosine, hypoxanthine etc. Two ADP molecules yield AMP in the adenylate kinase reaction (2 ADP ⇌ ATP + AMP), giving a quadratic relation between AMP production and ADP concentration (*35*):

 (7)

In long experiments there is significant resynthesis of ADP, V­synth,ADP, accompanied by reuptake of adenosine, inosine, hypoxanthine etc. (*31, 32*), phenomenologically represented by :

 (8)

with AdNinitial­ set as the target level for resynthesis.

Intracellular storage of carbon metabolites, glcstore, taken from the glycolytic intermediate pool and used for synthesis and growth, occurs with rate Jstore (unit amount of 6-carbon equivalents per liter intracellular water per second):

 (9)

Storage of glucose is appreciable and is required to close the mass balance of measured metabolites (Figure 2). Jstore may be assumed to be proportional to the rate of cell mass increase during growth. Jstore = 10 μM/s corresponds to a growth rate of 0.03 g glucose incorporated in the metabolite pool per gram dry mass of the cell per hour.

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|  |  | percent |

Algebraic Relations

Net ATP synthesis by glycolysis, JATP,glyc, is the ATP synthesis by the tail section minus the ATP consumption by the head section:

 (10)

Note that J­­head is given in 6-carbon units and Jtail­­ in 3-carbon units.

The total amount of NAD + NADH, NADtot, is assumed constant in the model and NADH concentration is calculated from the NAD concentration.

 (11)

Oxygen consumption is related to the mitochondrial ATP synthesis by the P/O2 ratio:

 (12)

Three O2 molecules correspond stoichiometrically to oxidative metabolism of one lactate molecule. Three O2 also correspond to the combination of pyruvate and NADH + H+ which is produced in a one-to-one ratio either in the tail end of glycolysis or from lactate. The mitochondria are assumed to consume pyruvate at rate Jpyr,mit in stoichiometric ratio to their O2 consumption:

 (13)

The mitochondria consume NADH + H+ at rate Jnadh,mit via redox shuttling (*36*) and produce nicotinamide adenine dinucleotide (NAD):

 (14)

Oxidation of the pyruvate molecule by itself corresponds stoichiometrically to 2 ½ molecule O2 and NADH+H+ to ½ molecule O2. The factor 3 in eqs. 13 and 14, however, reflects the simplifying assumption that the mitochondria metabolize one NADH per pyruvate taken up.

FBP normally forms the vast majority of PGI and represents PGI in the model. This is efficient because FBP can be directly compared to measurements. The total PGI content has a fixed ratio, RPGI/FBP, to the FBP content in the model (*5, 6, 26*) :

 (15)

In the mass balances of glucose-derived metabolites the complete PGI content is taken into account.

Differential Equations

Glucose is used by the head section of glycolysis and is assumed to be distributed at equal concentrations in the cytosolic and extracellular (interstitial) volumes of distribution which is a reasonable approximation given the high glucose transport capacity of the EATC cell membrane (*37*) :

 (16)

Vcyt is cytosolic water volume as fraction of the total intracellular water volume; Vext is the ratio of extracellular water volume to total intracellular water volume. Eq. 16 gives the rate of change of glucose concentration in cytosolic and extracellular water. Note that concentrations are given in mol per liter H2O in the respective compartments (for glucose the cytosol and extracellular volume) and metabolic fluxes J are in mol per liter total intracellular H2O per s. Expression of flux per volume of total cell water provides a common reference.

Lactate is produced from pyruvate and NADH by the lactate dehydrogenase reaction and distributed in the cytosolic and extracellular space:

 (17)

The reaction is reversible and, with Jlac negative, lactate and NAD can be converted to pyruvate and NADH and be used for mitochondrial metabolism.

Pyruvate is produced by glycolysis and used for lactate production and mitochondrial metabolism:

 (18)

Lactate and pyruvate are distributed intracellularly and extracellularly at equal concentration to keep the model as simple as possible.

The time-dependent ATP balance is the result of the net glycolytic ATP synthesis rate, see Eq. 10, mitochondrial ATP synthesis (Jmit) and hydrolysis for cellular processes and growth (Jhyd,ATP):

 (19)

ATP, like other phosphorylated metabolites such as ADP, FBP and other PGI, does not cross the cell membrane and is distributed intracellularly only.

In addition to ADP’s involvement in ATP usage and resynthesis, ADP is also broken down at rate Jbreakdown,ADP to AMP, adenosine, inosine, hypoxanthine, etc., and resynthesized at rate Jsynth,ADP (*31, 32*):

 (20)

The fructose 1,6-bisphosphate (FBP) concentration represents the total phosphorylated glycolytic intermediate pool, of which it forms the major part. FBP rather than PGI is used as state variable because it is directly measured in the experimental data sets. FBP is related to PGI by a constant ratio (Eq. 15). FBP’s rate of change is the result of synthesis by the head section of glycolysis, consumption by the tail section of glycolysis and uptake of glycolytic intermediates in the cell in various molecular forms for storage and growth:

 (21)

The balance of the fluxes gives the rate of change of the total PGI pool; division by RPGI/FBP results in the rate of change of FBP. The factor ½ before Jtail takes into account that J­­tail­ represents flux of 3-carbon units while FBP contains 6 carbon atoms.

The head section of glycolysis is inhibited by signals from the phosphorylated glycolytic intermediate pool (see above: *Description of the computational model*), represented by FBP in the model. The active fraction of the head section is denoted by Factive.

 (22)

The second order forward rate constant for inactivation, k­f, and the backward rate constant from the inactive state, kb, represent the delay of the inhibition and the reactivation time courses of the head section, respectively.

The concentrations of nicotinamide adenine dinucleotide (NAD) and its reduced form (NADH) depend on the balance of the fluxes which oxidize and reduce them:

 (23)

NAD and NADH in turn affect Jtail, Eq. 2, and Jlac, Eq. 4.

Transport Equations for Diffusion and Blood Flow in Tissue Containing Two Cell Types

Transport by blood flow and diffusion is modeled with a simple geometry: oxygen and glucose are delivered by flow in a blood vessel which is surrounded by a radially symmetric cylinder of tissue where molecules diffuse and are metabolized. Transport and metabolism of lactate and pyruvate are also taken into account. Transport in tissue is by diffusion. The tissue contains two separate cell types with distinct metabolic characteristics: each cell type has its own distinct intracellular metabolite concentrations and has a separate set of differential Eqs. 16-23 for metabolism.

Vascular networks in tumor tissue appear chaotic with asymmetric structures and heterogeneous, long and short flow pathways (*38*). This leads to shunting of blood flowing via short pathways, diffusional shunting between adjacent arterial and venous sections of the microcirculation, and complex oxygen profiles with broad distributions of tissue oxygen tension (*39*). Rather than simulate any specific complex tumor vascular network geometry, the aim here is to simulate a simple geometry which reveals basic principles of how fluctuating blood flow causes cycling nutrient and oxygen levels. The model addresses how diffusion gradients influence oxygen and nutrient supply and shows what the metabolic response is of tumor cells with the “Warburg effect” glycolytic phenotype under fluctuating nutrient conditions in tissue.

The oxygen concentration immediately adjacent to a small blood vessel in tumor tissue, O2,perivascular, is determined in the model by the balance of oxygen carried by the blood into the microvessel at the arterial side, leaving the microvessel via the venous efflux, and diffusing across the vessel wall into the surrounding tissue:

 (24)

Variables and parameters in the equations are described in Supplementary Tables 1-3.

For the oxygen dissociation curve of hemoglobin an empirical model is used: the Hill equation cn/(cn+c50n) with Hill coefficient n=2.6 and c50, the concentration at which hemoglobin’s oxygen binding sites are 50% saturated with O2 (*40, 41*). The c50,O2 is specified separately for arterial and venous blood mainly because of the pH difference. In Eq. 24 cv,O2 is set equal to the perivascular O2 concentration, which is a simplifying assumption. In tumor tissue a whole range of oxygen saturations in the microvessels is observed (*42*). O2,perivascular represents the oxygen concentration in the outflow region of an “average” microvessel, which is neither in a short shunt pathway for blood flow, nor in a very long pathway where hemoglobin oxygen saturation becomes virtually zero. The present simplifying approach is useful to demonstrate the metabolic response of tumor cells with high and low glycolytic capacity to fluctuating supply conditions that occur in tumor tissue, but should not be taken to represent an accurate O2 concentration profile for a specific tumor tissue including very heterogeneous perfusion and diffusion pathway lengths.

Diffusion across the microvessel wall is given by Fick’s diffusion law, taking the diffusion coefficient DO2, the concentration gradient of oxygen at the vessel wall and the area available for diffusion into account:

 (25)

with r­­vessel the radius of the blood vessel and r the radial distance into tissue. Note that the diffusion flux JO2 is given per volume of intracellular water in the area supplied by the vessel. The cylinder length of the considered volume, Lcyl, which determines the surface area of the blood vessel available for diffusion, is therefore also normalized per unit intracellular water volume. This maintains the principle of a common reference to be able to compare transport and metabolic flux values.

The rate of change of oxygen concentration in tissue is determined by local oxygen diffusion and by the oxygen consumption of two different cell types:

 (26)

The two cell types have distinct Vmax parameters for equations 1 and 2, representing different glycolytic capacities, and make up a fraction f­cell type 1 and f­cell type 2 of the tissue. Vext represents the extracellular (interstitial) volume as fractional value of the intracellular water volume in the same tissue layer.

The tissue is represented by concentric layers in a cylindrical geometry. A segment (layer) of the cylinder is defined by inner radius rinner and outer radius router. A tissue cylinder to calculate diffusion is simulated by a number N of concentric cylindrical layers, one fitting inside the other, with increasing rinner and r­outer and fixed width router - rinner. The net oxygen diffusion, Jdiff,O2, into a tissue layer is determined by Fick’s diffusion law for cylindrical geometry and expressed per unit volume of intracellular water contained in the layer. Lcyl is the length of the cylinder per unit volume of intracellular water contained in the same layer.

 (27)

The rate of change of the diffusing metabolites, M, in the model (glc=glucose, lac=lactate, pyr=pyruvate) in the space in and immediately around the blood vessels is given by :

 (28)

The equation for diffusion of metabolite M across the vessel wall, Jdiff vessel wall M­, is the same as Eq. 25 with M replacing O2.

The rate of change of the diffusing metabolites in a tissue layer is given by:

 (29)

Here JM,1 and JM,2 are the uptake rates of the metabolite in the two cell types (production is represented by negative values). Uptake of glucose equals Jhead­ and uptake of lactate –Jlac­; pyruvate uptake equals Jlac + Jmit,pyr - Jtail.

The net diffusion into the tissue layer is given by:

 (30)

Simulating tumor tissue with fluctuating blood flow

Blood flow in tumor vessels is often fluctuating or intermittent, e.g. (*43-47*). Fluctuating blood flow was simulated by adding a sinusoidal component with amplitude Aflow and period Tperiod to a constant offset Oflow :

 (31)

Fblood = Oflow for t<0. Blood flow is only allowed in one direction by imposing zero as lower bound on flow. Consequently, a period of zero blood flow results if the amplitude of the sinusoidal function is larger than the offset. Blood flow levels were taken in the range measured for cancer tissue (*48, 49*).

The conditions in Figures 3 and 4 and Supplementary Figures 1-4 lead to low tissue oxygen concentration due to low and fluctuating blood flow which is characteristic of cycling hypoxia. Chronic hypoxia, in contrast, often results from large diffusion distances in tissue (*44*). The maximal diffusion distance in tissue was set to 40 μm from the blood vessel, as found for the supply region from the blood vessel in the experiments on implanted tumors (*48*) which were used as the example condition for the tissue simulations (see main text). For these distances in tissue, diffusion limitation has often only a modest influence, and hypoxia is mainly of acute nature due to cycling blood flow. No diffusion of oxygen across the 40 μm boundary of the tissue region supplied from the microvessel was allowed in the calculations. This is the non-flux boundary condition often used in tissue simulations of diffusional transport. For Ehrlich ascites tumor cells being grown in the peritoneal cavity, among others in Warburg’s laboratory, a maximal diffusion distance of 630 μm was used for the simulations (see below). In this case severe hypoxia results from diffusion gradients.

In Figures 3 and 4 and Supplementary Figures 1-3 (see Dataset 4) blood flow was constant for t<0 at 0.264 ml blood per ml intracellular H2O per min (11 ml/100g tissue/min) upon which an amplitude of 0.264 ml/ml/min was superimposed to simulate sinusoidally fluctuating blood flow. Cell type 1 constituted 80% of the cells and had the full tumor glycolytic capacity as found in EATC (Figure 2); cell type 2, the remaining 20%, had a glycolytic capacity of 10% of the EATC level. In Supplementary Figure 3 cell type 2, 20% of the cell volume, had a glycolytic capacity of 1.5% of the EATC level. Results in Figures 3, 4 and Supplementary Figures 1-3 are for the tissue cylinder segment at 15-20 μm from the blood vessel (*48*).

Simulations of tumor cells in tissue during two minute flow stops with ATP hydrolysis less sensitive to decrease of energy status are detailed below.

Computational Methods

Programs to calculate computational model results and optimize parameters were written in the R computational language (*50*) and are available under an open source license. The system of ordinary differential equations was integrated using the lsoda algorithm implemented in R package deSolve (*51*). This algorithm switches methods adaptively to solve stiff and non-stiff equation systems (*52*). Diffusion fluxes in tissue or ascites fluid were calculated using a finite difference scheme (*53*). The computer code implements diffusion equations in cylindrical geometries with a variable number of layers and builds the concentrical layer structure dynamically. For simulations of tissue *in vivo* eight layers of width 5 μm were used. Parameter optimization procedures based on experimental data and analysis of prediction uncertainty are treated below.

Parameter Optimization

The cost function used for the optimization of model parameters was obtained by adding the squares of the differences between calculated model results and measurements in experiments 1-3 (see below), weighted by estimates of experimental error. Measured data points are given in Figure 2. The steady state ratio of lactate production to oxygen consumption during one hour after addition of a high concentration of glucose from experiment 3 (see below), measured in Warburg’s laboratory (*3*), provided an additional data point. For the one hour challenge with glucose, the FBP concentration was found experimentally to decline <50% from 5 min onward and to attain stable values after 10 minutes (*54, 55*): squared changes of FBP after 10 min and decrease of FBP levels below 50% were added to the cost function. Model parameters were estimated using a Nelder-Mead (simplex) optimization procedure (*56*) by minimizing the cost function. Results of the parameter optimization are given in Supplementary Table 3. With the estimated parameters, the behavior of the ascites tumor cells in experiments 1-3 was reproduced. Figure 2 shows the fit of the model to the data. In the present study, seventeen parameters were estimated by the Nelder-Mead method, based on 83 data points. This is a relatively large number of parameters. In the next section the question is addressed whether predictions based on model simulations with the estimated parameters are sufficiently well-constrained.

Parameter Sensitivities and Analysis of Prediction Uncertainty

Systems biology models often have many poorly known parameters and “sloppy” parameter sensitivity spectra (*57*). This situation can be meaningfully approached with statistical mechanical methods (*58*). It has been shown that predictive models of the behavior of intact biological systems are usually well-constrained if joint parameter sets for systems biology models are obtained by collective fitting. Markov chain Monte Carlo (MCMC) methods to accomplish this take the (nonlinear) dependencies between the estimated parameters into account (*57*). By using parameters sets from collective fits, this approach can even give good predictions if many or even all individual parameter estimates are rather imprecisely known. Well-constrained predictions were for instance obtained after fitting 59 parameters of a model of a complex signaling network to 68 data points (*59*). Such a strategy to deal with “sloppy” parameter sensitivities was shown to be universally applicable to a large number of systems biology models (*57*), and was previously applied in our laboratory to investigate the creatine kinase energy buffering system in heart (*21*) and skeletal (*60*) muscle.

To assess the uncertainty range of likely parameter distributions around the Nelder-Mead optimized point, different parameter combinations for the present model were generated with an MCMC method, taking the experimental noise in the data into account. The starting point for the Markov chain was the optimal parameter set from the Nelder-Mead method and the same cost function was applied as for the optimization. The collection of parameter sets thus obtained reflects the likelihood of the parameter sets given the experimental data and also reflects correlations and nonlinear dependencies amongst the parameters. For the MCMC approach an adaptive Metropolis-within-Gibbs algorithm was applied (*61*), implemented in R package spBayes (*62*). Forty five Markov chains of 14000-16500 samples each were generated with independent random seeds; each MCMC run took 4-5 days, close to the maximum time allowed on the Lisa cluster computer (see Acknowledgements). Taking the last member of each of the 45 MCMC chains yields parameter sets representing the likelihood of the parameter combinations given the measured data. According to the Heidelberg-Welch statistic the chains tended to converge before the end of the runs and the chains were long relative to the spectrum of autocorrelation times determined with R package “coda” (*63*). Some parameters were relatively precisely determined based on the data, while the ranges for other parameters were sometimes very broad. The interquartile range for the optimized parameters determined from the 45 replicated MCMC files are given in Supplementary Table 3. The quantiles were calculated according to definition 7 in (*64*), which is the default in R. Some of the parameters were determined quite well, while others were rather imprecisely determined. Nevertheless, the model response to the experimental interventions of Figure 2 varied little over the 45 parameter sets (Supplementary Figure 2). This demonstrates that model behavior is well constrained, despite the variation in the precision of parameter determination, as previously shown for other systems biology models (*57*).

The 45 parameter sets obtained by MCMC were then used to repeat the simulations of metabolic responses in tissue in Figure 4, revealing the variation of predictions of system behavior based on the spectrum of estimated model parameter sets (*21*). Supplementary Figure 1 shows the median and the 5 and 95% quantiles of the predictions from the MCMC ensemble. It is clear that the prediction of the metabolic response to blood flow oscillations given in Figure 4 is sufficiently constrained: for each of the MCMC parameter sets the FBP buffering of ATP is much weaker in the cells with the 90% reduced glycolytic capacity compared with cells with the full tumor glycolytic capacity.

Calibrating the computational model with experimental data

A data set was assembled consisting of representative experiments to estimate the model parameters for the predictive model of metabolic responses of Ehrlich ascites tumor cells. These data sets from the scientific literature are exemplary, but they are representative of results measured in many laboratories (*1, 3-9, 25, 26, 28, 54, 65-69*). All selected experiments were done at 37 oC on Ehrlich ascites cells which had been grown in mice. During the experiments aerated tumor cell suspensions were diluted in buffer solution. Cells and suspension had been depleted of glucose for some time and were respiring on endogenous substrates such as lactate, which was abundantly present. At t=0 glucose was added to the suspension. Experimental methods were described in detail in the original publications. The following experiments were included in the calibration data set:

Experiment 1. In this experiment 3.5 µmol glucose was added per ml of cell volume to an Ehrlich ascites cell suspension in which glucose was depleted and which was respiring on endogenous substrates, mainly lactate, present in the suspension (*6*). The glucose addition resulted in a start concentration of 92 µM glucose. Cell volume fraction was 2.2%. Amount of glucose taken up and FBP and ATP concentrations were measured at 0, 10, 20, 30, 45, 60, 90, 120 and 180 seconds after glucose addition and included in the data set. (The lactate measurements reported for this experiment were not included because they were later reported to be imprecise by the same research group (*26*).)

Experiment 2. Here 26.6 µmol glucose was added per ml of cell volume, resulting in a start concentration of 776 µM glucose at a cell volume fraction of 2.9%. Before glucose addition, cells were metabolizing endogenous substrates (*26*) including lactate. Amounts of glucose taken up, FBP and ATP concentrations and amount of lactate produced were measured at 0, 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, 240 and 300 seconds after glucose addition and were included in the data set. Oxygen uptake measurements with an oxygen electrode were also included in the data set. ATP production before glucose addition was ~199 µM/s, based on respiration. The calculated mass balance of the experimental data (see Figure 2) on phosphate metabolites (ATP and FBP), lactate production (1 lactate molecule set equivalent to 1 ATP synthesized) and respiration showed that 15 sec after glucose addition ATP hydrolysis had decreased to around a third of the initial value, and recovered to above 100 µM/s after 4-5 min. Analysis of the data showed that ATP hydrolysis correlated with the sum of ATP and ADP rather than with ATP or ATP/ADP. ATP hydrolysis was approximately linearly related to the sum of the ATP and ADP concentrations. The calculated balance of glucose taken up and accumulated amounts of FBP, lactate, pyruvate and pyruvate consumption by the mitochondria, showed that by 300 s almost one third of the carbon atoms in the sequestered glucose was taken up in intracellular metabolite stores. Intracellular storage of carbon, see Eq. 9, originating from the glucose taken up, is therefore required to obtain a carbon balance in the model agreeing with the experimental data.

Experiment 3. Experiments in Warburg’s laboratory reported by Tiedemann (*3*). The uptake of oxygen and production of lactate during one hour after addition of 11.1 mmol glucose per liter to a 0.43% suspension of Ehrlich ascites tumor cells was measured. In this experiment the cells were suspended in salt-containing buffer without amino acids. Under these conditions protein synthesis in EATC decreases within the first 5-10 min and glucose from the medium is depleted at a reduced rate (*70*). Storage of glucose in the model calculations was therefore reduced if it exceeded the level obtained after this first period.

Testing the computational model with additional experimental data

Model predictions were compared with five experimental data sets that had not been used in the parameter estimation:

Experiment 4. Experiments where different amounts of glucose were given. Model predictions were compared with the measurements to test the dependence of model results on glucose concentration. EATC respiring on endogenous substrates were given ten different amounts of glucose at t=0 (across five separate experiments) resulting in initial glucose concentrations between 29 and 800 μM. The maximal FBP accumulation was measured (*6*). In the simulations the maximal FBP concentration was predicted for each amount of glucose and compared with the experimental data.

Experiment 5. Fast metabolic responses in the first ten seconds were examined. Start concentrations of 77 and 154 µM glucose were given at t=0 to EATC which respired on endogenous substrates before that time. The FBP accumulated and the lactate produced in 5 and 10 sec were measured (Figure 6 in reference (*26*)). This agrees with the simulations (see Dataset 1, worksheets `Exp 5` and `Accumulation in 5 and 10 sec exp 5`).

Additional support comes from data in Saha and Coe (*37*) (see Table I in that reference) on measured glucose uptake and FBP accumulation after 10 s for a range of added glucose concentrations (0.2-10 mM). Both computer simulations and experimental results showed saturation of the FBP accumulation above 400 µM glucose.

Experiment 6. Anaerobic metabolism measured by Tiedemann in Warburg’s laboratory. As in experiment 3 an initial concentration of 11.1 mM glucose was added to a 0.43% cell suspension at the start of the measurement. Hydrogen cyanide (HCN) had also been added, which inhibits mitochondrial oxygen consumption (*3*). In the simulation oxygen consumption was also inhibited. Oxygen uptake and lactate production with and without HCN were compared between experiment and simulation. Simulation with and without mitochondrial oxygen consumption active are given in Datasets 1 and 2, respectively. Note that this experiment was reported in the same paper as Experiment 3.

Experiment 7. Effect of pyruvate on metabolite accumulation after glucose addition. ATP levels decline appreciably in simulations and measurements when glucose is added, but when 5 mM pyruvate is added at the same time as glucose, the decline of ATP is reduced to merely 0.1% in the simulation, as opposed to 30% when the concentration of pyruvate is low. This is similar as the pattern seen in experiments on ascites tumor cells (*31*). Simulation results are given in Dataset 3.

Experiment 8. Effect of addition of glucose on respiration. O2 consumption of EATC during one hour was measured in Warburg’s laboratory, comparing 0 and 11 mM glucose in the medium. The reduction of respiration, known as the Crabtree effect, was assessed (*3*). Note that this was reported in the same paper from Warburg’s laboratory as Experiments 3 and 6.

The relatively small computational model adequately represents these eight experimental data sets selected from the literature. These data sets are representative of results repeatedly measured in many separate laboratories, e.g. (*1, 3-5, 7-9, 15, 16, 19, 24-28, 30-32, 36, 37, 55, 65-69, 71, 72*). Note for instance how Chance (*5*) reports a similar very high glucose uptake during the first minute after glucose addition followed by a strong decrease as simulated by the present model (Figure 2, Dataset 1). Initial accumulation of FBP is followed by a secondary decrease, and an initial dip in ATP concentration is followed by an increase to a level lower than the initial one (*5*). An initial stimulation of respiration is measured after glucose addition, followed by a decrease below the level measured before glucose (*4*). Despite its simplicity, the present model appears to adequately integrate a broad set of biochemical knowledge and experimental data on the metabolic behavior of ascites tumor cells. The model therefore appears suitable to simulate the metabolic response of ascites tumor cells and other tumor cells in tissue.

Simulation of tumor cells with low sensitivity of ATP hydrolysis to energy status and flow stops

In the simulations of Figures 3 and 4 and Supplementary Figures 1-3, the sensitive mechanism to adapt ATP hydrolysis to energy status plays an important role. To demonstrate how the powerful glycolytic machinery may protect the cell if this ATP hydrolysis reducing mechanism is less effective, a second hypothetical situation in tissue was investigated. The simulations in Supplementary Figure 4 (Dataset 5) focus therefore on the effect of dynamic ATP buffering by the tail section of glycolysis. To accomplish this, adenine nucleotide breakdown is not active in the simulation. The strong reduction of ATP hydrolysis with AdN = ATP+ADP, found in the in vitro experiments (Figure 2, Eq. 5), was replaced by weak reduction of ATP hydrolysis with decreasing ATP concentration. AdN is therefore replaced by ATP in Eqs. 5 and 6, and ADP is not counted. khyd,ATP = 0.00833 s-1 was chosen for the simulation in Supplementary Figure 4 and Dataset 5. This value is similar as measured for protein degradation, see Figure 7 in Gronostajski et al. (*33*). The ATPcutoff value was set to 1600 μM, khyd,AdN,low = 0 s-1, γ = 0.166 and Cpower = 23.51. Eqs. 5 and 6 now define a curve whose slope decreases with ATP or remains constant at high ATP.

The metabolic rate was kept relatively low, Vmax,hyd = 100 μM/s, and there was no storage of glucose-derived metabolites, corresponding with no growth of the tumor cells. For t<0 blood flow was constant at 0.132 ml blood per ml intracellular H2O per min (corresponding to 5.4 ml/100 g wet weight/min). Blood flow started to oscillate sinusoidally at t=0 with period 500 sec with amplitude of 0.21 ml blood per ml intracellular H2O per min (8.7 ml/100 g wet weight/min) while constrained to be >0 (top panel, Supplementary Figure 4). The first cell type, comprising 95% of the cells had the full tumor glycolytic capacity as found in EATC (Figure 2); cell type 2, the remaining 5%, had a glycolytic capacity as indicated above the rows in Supplementary Figure 4, which each represent separate simulations. The blood flow level and minimal growth are similar to conditions in old implanted tumors (*48*). The effect of the ATP buffering by FBP remains and its effect can therefore be assessed in isolation.

Although the average blood flow for t>0 was slightly higher than for t<0, ATP levels declined at low and stopped flow in the cells with decreased glycolytic capacity. Results in Supplementary Figure 4 were for the tissue layer at 35-40 μm from the blood vessel. As a result of the small sensitivity of ATP hydrolysis to energy status there was a large decline of ATP when glycolytic capacity was only modestly lower than in tumor cells (Supplementary Figure 4). The simulations show that FBP buffering supported by the full tumor glycolytic capacity will protect the cell also when adaptation of ATP hydrolysis is not effective. In contrast, modest reductions in glycolytic capacity lead to decreased ATP concentrations and hydrolysis during the low flow period. When ATP synthesis from the FBP buffer is uncoupled such that no ATP synthesis is accomplished, ATP levels fall dramatically even when glycolytic capacity is high, but only when blood flow fluctuates (Supplementary Figure 4, bottom row).

Simulation of glucose metabolism of Ehrlich tumor cells in ascites fluid

Warburg and Hiepler (*72*) noted that glucose and oxygen contents were very low in the ascites fluid in which they were growing Ehrlich ascites tumor cells (EATC) in the peritoneal cavity of mice. They predicted from *in vitro* measurements that these cells should consume 44 mg glucose per hour per 5 ml ascites fluid if exposed anaerobically to saturating glucose concentrations *in vivo*. They surmised that this amount of glucose could not be provided by the host. Indeed, Kemp and Mendel (*73*) injected 5 ml ascites fluid not containing EATC intraperitoneally in mice and measured that at most 14 mg of glucose could diffuse into this fluid per hour. They also measured 5-7 mg/dl (280-390 μM) glucose concentration in ascites fluid containing growing EATC, sufficient for glycolytic rates very close to maximal values. This constitutes a paradox: the glucose concentration in samples of ascites fluid appears to be sufficient for high rates of glucose consumption by the tumor cells, but the transport capacity for glucose from blood into the ascites fluid is not high enough to sustain these high metabolic rates. To resolve this paradox, Kemp and Mendel suggested that in the presence of proliferating ascites cells *in vivo*, the diffusion gradient for glucose would be kept higher because the cells consume glucose at a high rate. They hypothesized that this would allow sufficient glucose diffusion when EATC metabolize glucose at near-maximal rates. Kemp and Mendel proposed further that the liver of the murine host resynthesizes glucose from the lactate generated by the EATC to sustain the high glucose metabolism in the ascites tumor cells. It is of some historical interest that Warburg endorsed their explanation in a note added retrospectively to the Hiepler and Warburg (*72*) paper of 1952 when this paper was reprinted in a collection volume in 1961(*74*).

Burgess and Sylvén (*75*) also measured a low glucose concentration, around 300 µM in ascites fluid where EATC were growing. They found glucose concentrations of ~145 mg/dl (~8 mM) in normal peritoneal fluid not containing EATC. Note that mixing of glucose-depleted ascites fluid with a small amount of peritoneal fluid from parts of the peritoneal cavity which do not contain glycolytically active EATC might therefore cause average glucose concentrations in sampled ascites fluid above 200 µM.

The hypothesis is now investigated here that diffusion gradients for glucose in the ascites fluid containing tumor cells are large. The peritoneal surface in mice is estimated to be 79 cm2 (*76*). For a peritoneal fluid volume of 5 ml (*73*) this means that the peritoneal surface can be thought of as being covered by a fluid layer of ~630 µm on average. Although this is a simplified approximation to a much more heterogeneous situation, diffusion distances are probably large for a substantial part of the ascites fluid. Consequently, glucose concentrations may become limiting for glucose uptake of the cells in a major fraction of the ascites fluid. This is illustrated here by simulation with the model for metabolism (Eqs. 1-23) including diffusion from blood vessels into the ascites fluid containing the tumor cells (Eqs. 24-30).

First the experiment of Kemp and Mendel (*73*) to measure the glucose transport capacity into ascites fluid without cells is simulated: 5 ml of cell-free ascitic fluid, containing 3 mg/dl (~167 µM) glucose, was injected into the peritoneal cavity of mice. Due to diffusion, the average ascites glucose concentration increased to 17 mg/dl (944 µM) in 3 min. A simulation of a 630 µm layer of ascites fluid on the peritoneal membrane showed a similar increase in average glucose concentration (Dataset 6). With tumor cells (25% of the volume) in the ascites fluid and the same glucose supply conditions, glucose consumption by the simulated EATC is ~49 µM/s at diffusion distances up to 100 µm from the peritoneal blood vessels. This increases to ~78 µM/s at ~170 µm because the O2 concentration has fallen close to zero and anaerobic glycolysis supplies the ATP (Dataset 7). Beyond 300 µm diffusion distance from a blood vessel, glucose and O2 concentrations in the ascites fluid are both so low that cellular glucose uptake and ATP production become zero; this applies to the larger part of the simulated ascites fluid volume. At the same time the average glucose concentration is ~119 µM, which is more than twofold the Km value for glucose. The average cellular glucose uptake, ~13 µM/s, in the ascites tumor cells in the simulation of the situation *in vivo* is much lower than for Warburg’s *in vitro* measurements (*72*) which yielded ~55-68 µM/s under anaerobic conditions and ~35 µM/s under aerobic conditions (*3*). The average uptake of 13 µM/s corresponds with ~9 mg per hour per 5 ml which is within the diffusion capacity reported by Kemp and Mendel, see above (*73*). Cellular glucose uptake in the ascites fluid is probably very heterogeneous because of the large diffusion gradients. Perfusion heterogeneity in the peritoneum may cause additional variation. According to the simulation results, a substantial fraction of cells at diffusion distances 130-300 µm from blood vessels are exposed to a combination of O­2 < 0.1 µM with glucose still present, which results in anaerobic glycolysis.

The tumor cells in the ascites fluid are expected to change position relative to the blood vessels in the peritoneum because of intestinal peristalsis, muscle contraction and body movement (*77*). Injected dye spreads through the peritoneal cavity of mice in 2-4 min (*78*). Movements of cells relative to the peritoneal blood vessels were simulated in two ways. In the first simulation approach, the concentration of metabolites was set constant at t=0 throughout the fluid volume at average values and the relaxation of concentration towards the steady-state diffusion gradient was simulated for t>0. According to these simulations O2 concentrations typically adjust upward in 1-2 s with initial rate 50 μM/s, while downward changes take longer at initial rate <10 μM/s. Increases of glucose concentration take ~20 s at initial rate 500 μM/s while a downward change takes >1 min at initial rate ≤15 μM/s. Downward changes in concentration in deeper layers further removed from blood vessels affect the upper layers with delay and diminished amplitude, causing two phases in the response. The faster changes for oxygen are related to the lower concentrations relative to glucose.

The second simulation approach to investigate the speed of adaptation of concentrations to displacement of fluid started from the steady-state diffusion gradient (found in Dataset 7) and fluid was moved at t=0 from 200-400 μm from the blood vessel to 0-200 μm and at the same time fluid from 0-200 μm was moved to 200-400 μm. Oxygen concentration in ascites fluid moving close to a peritoneal blood vessel increases within 2 s from <0.1 μM to above 90 μM and glucose concentration from 100 to 4800 μM. A downward change in oxygen concentration in fluid moving away from the vascular oxygen source is virtually complete in 2 s, but the decline of a high glucose concentration takes longer. In this second type of simulation the concentration changes were secondarily affected by changes in metabolism throughout the ascites fluid. Cells moving quickly closer to a blood vessel from a distance >200 μm, largely depleted of FBP, show a response similar as in Figure 2, with strong initial glucose uptake, FBP accumulation and brief stimulation of oxygen consumption.

In short, simulations suggest that a large fraction of the ascites fluid contains very low O2 and glucose concentrations. The movement of fluid and cells may therefore cause large fluctuations in nutrient supply. In this way the ascites fluid in the intraperitoneal cavity forms an environment for ascites tumor cells where a nutrient and energy buffering system to survive periods of nutritional shortage may provide a large competitive and selective advantage to the cells.

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