Journal of Bioenergetics and Biomembranes, Vol. 27, No. 5, 1995

Regulation and Control of Compartmentalized Glycolysis in Bloodstream Form *Trypanosoma brucei*

Barbara M. Bakker,[1,2] Hans V. Westerhoff,[1,2] and Paul A. M. Michels[3]

Received April 28, 1995

Unlike other eukaryotic cells, trypanosomes possess a compartmentalized glycolytic pathway. The conversion of glucose into 3-phosphoglycerate takes place in specialized peroxisomes, called glycosomes. Further conversion of this intermediate into pyruvate occurs in the cytosol. Due to this compartmentation, many regulatory mechanisms operating in other cell types cannot work in trypanosomes. This is reflected by the insensitivity of the glycosomal enzymes to compounds that act as activity regulators in other cell types. Several speculations have been raised about the function of compartmentation of glycolysis in trypanosomes. We calculate that even in a noncompartmentalized trypanosome the flux through glycolysis should not be limited by diffusion. Therefore, the sequestration of glycolytic enzymes in an organelle may not serve to overcome a diffusion limitation. We also search the available data for a possible relation between compartmentation and the distribution of control of the glycolytic flux among the glycolytic enzymes. Under physiological conditions, the rate of glycolytic ATP production in the bloodstream form of the parasite is possibly controlled by the oxygen tension, but not by the glucose concentration. Within the framework of Metabolic Control Analysis, we discuss evidence that glucose transport, although it does not qualify as the sole rate-limiting step, does have a high flux control coefficient. This, however, does not distinguish trypanosomes from other eukaryotic cell types without glycosomes.

KEY WORDS: *Trypanosoma brucei*; glycolysis; glycosome; flux control; Metabolic Control Analysis; diffusion.

INTRODUCTION

Trypanosoma brucei (Fig. 1) is a protozoan organism of the order Kinetoplastida. During part of its life cycle, this organism lives as a parasite in the bloodstream and other extracellular body fluids of mammalian hosts, where it obtains its free energy merely from glycolysis. The abundantly available glucose is converted at a high rate (Table I) into pyruvate, which is subsequently excreted. The exclusive dependence on glycolysis is found neither in other life stages of *T. brucei* nor in most other related protists.

Kinetoplastida share a unique organization of their glycolytic pathway. Those glycolytic enzymes that are responsible for the conversion of glucose into 3-phosphoglycerate (3-PGA)[4] reside in organelles called glycosomes (Fig. 2) (Opperdoes and Borst, 1977). The morphology, enzymic contents, and biogenesis of these organelles strongly suggest that glycosomes are evolutionary and, in part, functionally related to peroxisomes of other eukaryotes (Hannaert and Michels, 1994; Michels and Hannaert, 1994). In Kinetoplastida, peroxisomes are called glycosomes, because their predominant function is glycolysis.

In this paper we review the specific features of glycolysis as it occurs in bloodstream form *T. brucei* and address the question if compartmentation has

[1] E. C. Slater Institute, BioCentrum, University of Amsterdam, Amsterdam, The Netherlands.
[2] Microbial Physiology, BioCentrum, Free University, Amsterdam, The Netherlands.
[3] Research Unit for Tropical Diseases, International Institute of Cellular and Molecular Pathology, Brussels, Belgium.

0145-479X/95/1000-0513$07.50/0

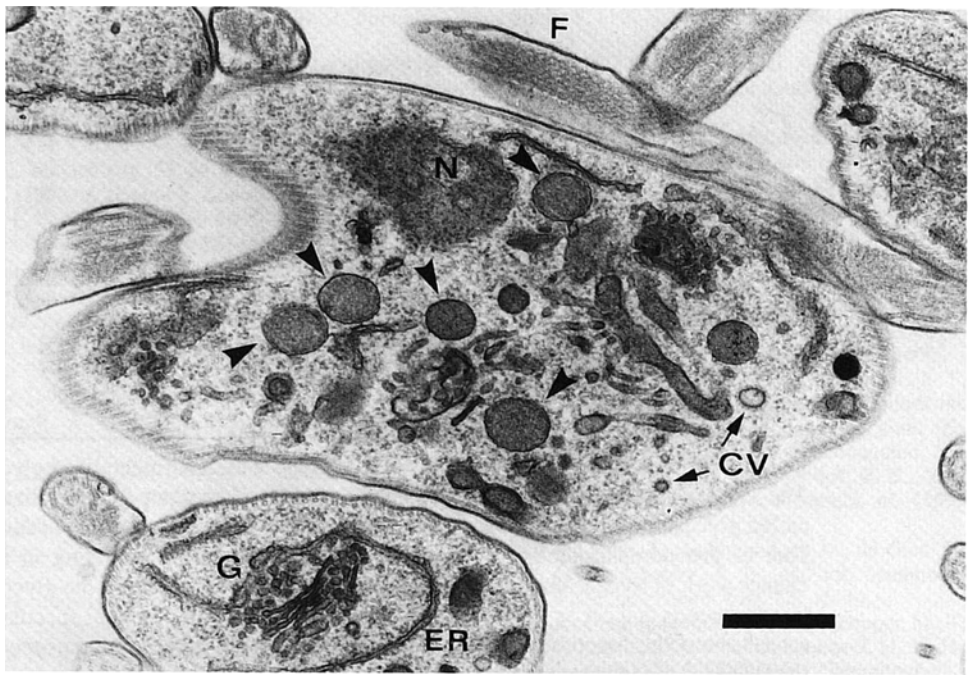

Fig. 1. Electron micrograph of a cross section of a bloodstream form *Trypanosoma brucei* cell. Glycosomes are indicated by arrowheads. N, nucleus; F, flagellum; CV, coated vesicles; G, golgi apparatus. ER, endoplasmic reticulum. The bar indicates 0.5 μM. (By courtesy of Dr. I. Coppens, ICP, Brussels.)

important consequences for regulation and control of this metabolic pathway.

GLYCOLYSIS IN BLOODSTREAM FORM *Trypanosoma brucei*

Some glycolytic enzymes occur not only in the glycosome and some are even represented in the cytosol by a different isoenzyme. Yet, pulse-labeling studies on intact *T. brucei* cells with ^{14}C-glucose have shown that the glycolytic flux goes virtually exclusively through the glycosome (Visser *et al.*, 1981).

[4] Abbreviations used: AK: adenylate kinase; ALD: fructose-1,6-bisphosphate aldolase; 1,3-BPGA: 1,3-bisphosphoglycerate; C: control coefficient; DHAP: dihydroxyacetone phosphate; ϵ: elasticity coefficient; e_i: activity of enzyme *i;* ENO: enolase; F-1,6-BP: fructose 1,6-bisphosphate; F-6-P: fructose 6-phosphate; GA-3-P: glyceraldehyde 3-phosphate; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GDH: glycerol-3-phosphate dehydrogenase; GK: glycerol kinase; G-3-P: glycerol 3-phosphate; G-6-P: glucose 6-phosphate; GPO: glycerol-3-phosphate oxidase; HK: hexokinase; PEP: phospho*enol*pyruvate; J: flux; 2-PGA: 2-phosphoglycerate; 3-PGA: 3-phosphoglycerate; PGI: glucose-phosphate isomerase; PFK: phosphofructokinase; PGK: phosphoglycerate kinase; PGM: phosphoglycerate mutase; PYK: pyruvate kinase; TIM: triosephosphate isomerase.

Table I. Aerobic Glucose Consumption by a Variety of Intact Organisms and Tissues *in Vitro*

Organism or tissue	Aerobic glucose consumption rate (μmol min^{-1} per g wet weight)[a]
Saccharomyces cerevisiae	20–30
Pigeon retina	9–15
Ehrlich ascites tumor	11
Trypanosoma brucei	7
Schistosoma mansoni	1–5
Rat ascites hepatoma (H-91)	2
Rat skeletal muscle	
Tetanized	7
At rest	0.03
Guinea pig cerebral cortex	1.4
Rat heart	0.9
Rat liver	0.2
Human erythrocytes	0.05

[a] Modified from Shapiro and Talalay (1982), in which the references to the original data can be found. In addition, the value for bloodstream form *T. brucei* was calculated from data in Opperdoes (1987).

About 20–30% of the hexosephosphates and triosephosphates in the cell were labeled within 15 s upon the addition of radioactive glucose to the cells, while it took 14 min to label as much as 90% of these metabolites. Most probably, the rapidly labeled glycolytic

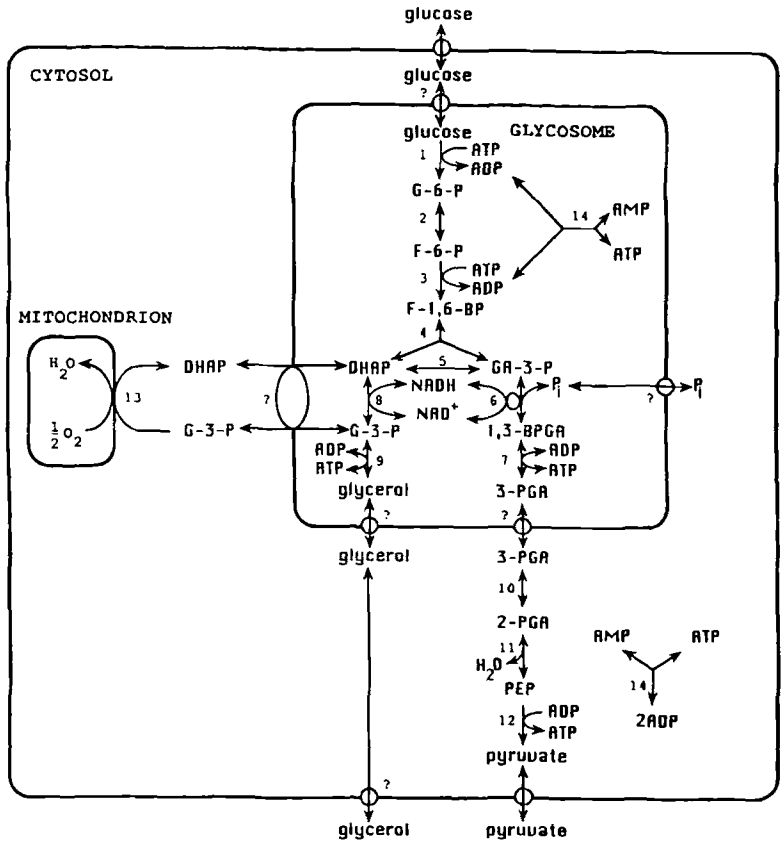

Fig. 2. Glycolysis in bloodstream form trypanosomes. Under aerobic conditions glucose is converted into pyruvate. Under anaerobic conditions equimolar amounts of glycerol and pyruvate are produced. Substrate and metabolite transporters in membranes are indicated by circles. Question marks indicate that the involvement of specific carrier molecules in transport across the membrane has not been unambiguously established until now. Enzymes: 1. HK; 2. PGI; 3. PFK; 4. ALD; 5. TIM; 6. GAPDH; 7. PGK; 8. GDH; 9. GK; 10. PGM; 11. ENO; 12. PYK; 13. GPO; 14. AK.

intermediates reside in the glycosomes, which constitute only 4% of the cell volume. The second pool of intermediates (70–80%), which appears not to be directly involved in glycolysis, represents metabolites present in the cytosol. The glycosomal membrane seems to be poorly permeable for the glycolytic intermediates. Therefore, it is likely that there are specific transport proteins in the glycosomal membranes for those metabolites that have to enter or leave the glycosome, such as glucose and 3-PGA (Fig. 2). Such transporters have, however, not yet been identified.

The NADH produced in the glycosome during the reaction catalyzed by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is reoxidized by a glycosomal glycerol-3-phosphate dehydrogenase (GDH). The redox equivalents are then transferred to a mitochondrial glycerol-3-phosphate oxidase (GPO) via a glycerol 3-phosphate/dihydroxyacetone phosphate shuttle (G-3-P/DHAP). It has been inferred from several data that this O_2-dependent alternative terminal oxidase is not involved in free-energy transduction (Grant and Sargent, 1960). However, this remains to be established unambiguously for more experimental conditions, especially since it was found that the electron transfer from G-3-P to O_2 involves ubiquinol/ubiquinone (Clarkson *et al.*, 1989; Bienen *et al.*, 1991) and that there is considerable variability in the H^+ per electron stoichiometry between and perhaps in terminal oxidases (see Van Spanning *et al.*, this issue). The G-3-P/DHAP shuttling mechanism guarantees a balanced redox state within the glycosome. Similarly, the consumption and production of ATP are balanced within the organelle. Net glycolytic ATP synthesis occurs only in the cytosol, notably in the reaction catalyzed by pyruvate kinase (PYK). Under anaerobic conditions or in the presence of salicylhydroxamic acid, an inhibi-

tor of GPO, the glycosomal G-3-P is converted into glycerol, with the concomitant production of ATP, by glycerol kinase (GK) (Opperdoes and Borst, 1977; Hammond and Bowman, 1980; Hammond *et al.*, 1985; Opperdoes, 1987). Consequently, the ATP and NADH balance within the glycosome are maintained also under anaerobic conditions. However, then one molecule of glycerol and one molecule of pyruvate are produced per molecule of glucose, instead of two molecules of pyruvate, resulting in only one molecule of ATP per molecule of glucose.

REGULATION OF GLYCOLYTIC ENZYMES

Kinetic analyses, using cell lysates and purified proteins, have shown that some glycosomal enzymes behave quite differently from their counterparts in other organisms. This is particularly true for hexokinase (HK) and phosphofructokinase (PFK) of which the activity is generally regulated by various glycolytic intermediates and heterotropic allosteric effectors. The trypanosomal enzymes appear to be largely insensitive to such effectors. No indications have been found for any regulation of HK activity: Glucose 6-phosphate, glucose, 1,6-bisphosphate, fructose 1,6-bisphosphate (F-1,6-BP), phosphoglycerates, phospho*enol*pyruvate (PEP), nor pyruvate exert any effect on the HK activity in a cell extract (Nwagwu and Opperdoes, 1982). Trypanosomal PFK has in common with its counterpart in other organisms that it shows positive cooperativity in the binding of its substrate fructose 6-phosphate (F-6-P). AMP and ADP stimulate the enzyme at subsaturating concentrations of F-6-P and are inhibitory at high concentrations of this substrate. PEP behaves as an allosteric inhibitor. However, the usual regulators F-1,6-BP, fructose 2,6-bisphosphate, citrate, and inorganic phosphate do not significantly influence the activity of this enzyme (Cronin and Tipton, 1985, 1987). In contrast, the activity of PYK, an enzyme located exclusively in the cytosol, is sensitive to a large variety of metabolic intermediates, most notably fructose 2,6-bisphosphate, which in other cells regulates the activity of PFK rather than PYK (Van Schaftingen *et al.*, 1985; Callens *et al.*, 1991a; Callens and Opperdoes, 1992). Consistently, the enzymes 6-phosphofructo-2-kinase and fructose-2,6-bisphosphatase are found in the cytosol (Van Schaftingen *et al.*, 1987). ATP, ADP, and AMP inhibit PYK by increasing its K_M for PEP. As inhibition by ATP is strongest and by AMP weakest (Callens *et al.*, 1991a), the activity of PYK decreases with increasing [ATP]/[ADP] ratio.

Many of the usual regulatory properties would be redundant for the glycosomal enzymes, because the low permeability of the organelle's membrane for most metabolic intermediates precludes feedback mechanisms usually operating in other cells. Moreover, in Trypanosomatidae activity regulation would not be necessary to control interactions between different pathways that share enzymes, for instance to prevent futile cycling at the level of PFK and fructose-1,6-bisphosphatase. Gluconeogenesis has never been demonstrated in bloodstream form *T. brucei*. In other Trypanosomatidae it is likely to be localized in the cytosol, as the pentose phosphate pathway, thus physically separated from glycolysis (Cronin *et al.*, 1989; Michels and Hannaert, 1994). This explains the insensitivity of PFK to fructose 2,6-bisphosphate.

Another striking consequence of compartmentation and the low permeability of the glycosomal membrane is that the glycosomal enzymes, among them HK and PFK, can be only indirectly sensitive to the cytosolic [ATP]/[ADP] ratio. This latter ratio may be very different from the glycosomal [ATP]/[ADP] ratio.

MOIETY-CONSERVED CYCLES

Sometimes overlooked, but very important for the analysis of metabolic pathways, are moiety-conserved cycles. These represent the sums of metabolite concentrations that remain constant on the time scale at which metabolic events occur, for example the sum of the redox coenzymes, NAD^+ plus NADH, and the sum of the adenine nucleotides, ATP plus ADP plus AMP.

As a consequence of compartmentation of glycolysis, three unique, moiety-conserved cycles appear in trypanosomes (Fig. 2). As in other organisms the cytosolic adenine nucleotide pool and the sum of NAD^+ and NADH are conserved. Unlike in other organisms there is an additional glycosomal adenine nucleotide pool and an additional glycosomal NAD(H) pool. Furthermore, the sum [G-6-P] + [F-6-P] + 2[F-1,6-BP] + [DHAP] + [GA-3-P] + [1,3-BPGA] + [G-3-P] + 2[ATP] + [ADP] within the glycosome is constant, if the transport of DHAP and G-3-P across the glycosomal membrane occurs via an exchange mechanism. The above sum can be seen as the total amount of transferable phosphate groups within the glycosome. In other organisms a similar conserved sum is not found, because ATP is hydrolyzed in free-energy

demanding processes. In trypanosomes it is, however, mainly the separate cytosolic ATP pool which is hydrolyzed. As one can also expect a low rate of hydrolysis of, for example, glycosomal ATP and 1,3-BPGA, the pool of transferable phosphate groups would be depleted slowly, unless there is a compensating influx of phosphorylated metabolites. Thus, a low permeability of the glycosomal membrane to metabolites contributing to this pool seems essential. We note that incomplete coupling of DHAP import to G-3-P export would relax this requirement.

CONDITIONS INSIDE THE GLYCOSOME

It has been postulated that the high glycolytic flux is possibly a result of compartmentation. This should enable either metabolite channeling or the occurrence of high intraglycosomal metabolite concentrations (Aman *et al.*, 1985; Misset *et al.*, 1986; Opperdoes, 1987). In principle, both metabolite channeling in the sense of direct metabolite transfer, also called reaction-diffusion coupling, and metabolite concentrations far above the K_M's of the enzymes, can increase the flux through a pathway, when this flux is not limited by the catalytic capacity of the enzymes, but by diffusion of metabolites from one enzyme to another.

The glycolytic enzymes are present at relatively high concentrations within the glycosomes of bloodstream form *T. brucei*. About 90% of the glycosomal protein content consists of glycolytic enzymes (Aman *et al.*, 1985) and the glycosomal concentrations of active sites vary between 0.055 mM for triosephosphate isomerase (TIM) and 0.5 mM for GAPDH (Misset *et al.*, 1986). The enzymes seem to be in close physical association, as can be inferred from the fact that they are only dissolved by addition of a high concentration of salt (0.15–0.25 M NaCl). Further evidence for close proximity of the enzymes was provided by cross-linking experiments (Aman *et al.*, 1985). Until now there is, however, no experimental evidence for metabolite channeling in glycosomes.

For the glycolytic intermediates of *T. brucei* high average concentrations have been measured: for several metabolites one order of magnitude higher than in most mammalian cell types (Visser and Opperdoes, 1980). Since 20–30% of these metabolites are present in the glycosomes (Visser *et al.*, 1981) which occupy only 4% of the total cellular volume, the concentrations in the organelles must be even higher: 0.5–7 mM (Table II) (Misset and Opperdoes, 1984). Meanwhile, most glycosomal enzymes have similar affinities for their substrates as their mammalian counterparts.

Westerhoff and Welch (1992) derived that diffusion is not likely to control glycolysis in yeast. This conclusion was based on the concentrations of glycolytic enzymes, hence on the average distance between the enzymes, and the intracellular viscosity. We performed analogous calculations for the glycosomal TIM and GAPDH. The mean-square displacement $(\Delta x)^2$ of a metabolite particle during a time period Δt resulting from three-dimensional diffusion is given by

$$(\Delta x)^2 = 6D\Delta t$$

With k_{cat} values of $1.1 \cdot 10^3$ s^{-1} (Lambeir *et al.*, 1987) for the conversion of DHAP into GA-3-P by TIM and $1.3 \cdot 10^2$ s^{-1} (Lambeir *et al.*, 1991) for the conversion of GA-3-P into 1,3-BPGA by GAPDH and a diffusion coefficient D of 10^{-6} cm^2 s^{-1}, this results in deplacements of 0.7 and 2 μm, respectively, during one turnover time of the enzymes. This is virtually sufficient for the metabolites to delocalize over the entire glycosome.

The concentrations of dimeric TIM and tetrameric GAPDH are 0.028 and 0.13 mM, respectively, corresponding to average enzyme–enzyme distances between nearest neighbors of $4 \cdot 10^1$ and $2 \cdot 10^1$ nm. If the trypanosomes would not have a glycosome and the same amount of glycolytic enzymes were diluted in the cytosol, i.e., in a 25 times larger volume, then the average enzyme–enzyme distances would be $1.2 \cdot 10^2$ and $7 \cdot 10^1$ nm for TIM and GAPDH, respectively. Thus, even for TIM, the glycolytic enzyme with the lowest concentration, the displacement of metabolites during one turnover time of the enzyme would still be 6 times as high as the average distance between the TIM molecules, if the enzyme were homogeneously distributed over the cytosol. Consequently, even if the glycolytic enzymes were not sequestered in a compartment, diffusion should not be controlling the glycolytic flux in *T. brucei*, unless the diffusion coefficient is substantially lower than the one for free diffusion assumed here. What is known about diffusion in cells suggests that small range diffusion is not more than threefold slower than free diffusion in water (Clegg, 1986). That long range diffusion may be limited by vermicelli in the cell soup, should not be relevant at the interenzyme distances calculated here (Westerhoff, 1985).

If a diffusion is not controlling the flux, one may ask what then causes the intraglycosomal metabolite

Table II. Glycolytic Metabolite Concentrations in Glycosomes of Bloodstream Form *T. brucei* Compared to Those in Mammalian Cells

		T. brucei[a]			Brain[i]			Muscle[i]		
		[S] (mM)	K_M (mM)	$K_M/[S]$	[S] (mM)	K_M (mM)	$K_M/[S]$	[S] (mM)	K_M (mM)	$K_M/[S]$
PGI	G-6-P	4.4	0.4[b]	0.1	0.13	0.21	1.6	0.45	0.70	1.6
	F-6-P	2.4	0.12[c]	0.050				0.11	0.12	1.1
PFK	F-6-P	2.4	0.85–1.9[d]	0.35–0.79				0.11		
	ATP		0.019–0.067[e]							
ALD	F-1,6-BP	1.9	0.009[f]	0.005	0.20	0.012	0.060	0.032	0.10	3.1
	GA-3-P	0.47	0.067[f]	0.14				0.003	1.0	$3 \cdot 10^2$
	DHAP	7.1	0.015[f]	0.002				0.050	2.0	$4.0 \cdot 10^1$
TIM	GA-3-P	0.47	0.25[g]	0.5				0.003	0.46	$2 \cdot 10^2$
	DHAP	7.1	1.2[g]	0.17				0.050	0.87	$1.7 \cdot 10^1$
GAPDH	GA-3-P	0.47	0.15[h]	0.32	0.003	0.044	$1 \cdot 10^1$	0.003	0.070	$2 \cdot 10^1$
	NAD		0.45[h]					0.60	0.046	0.080
	Pi									
	1,3-BPGA	0.77	0.10[h]	0.13	<0.001					
	NADH		0.02[h]							
PGK	1,3-BPGA	0.77			<0.001	0.009	>9	0.06	1.2	$2 \cdot 10^2$
	ADP				1.5	0.070	0.046	0.60	0.35	0.58
GDH	DHAP	7.1						0.050	0.19	3.8
	G-3-P	5.2						0.22	0.19	0.90

[a] The intraglycosomal concentrations in *T. brucei* are based on experimental data reported by Visser and Opperdoes (1980) and calculated as described by Misset *et al.* (1986).
[b] B. M. Bakker, estimated from unpublished results.
[c] Marchand *et al.*, 1989.
[d] Cronin and Tipton, 1985.
[e] Cronin and Tipton, 1987.
[f] Callens *et al.*, 1991b.
[g] Lambeir *et al.*, 1987.
[h] Lambeir *et al.*, 1991.
[i] The data for mammalian cells were taken from Fersht (1985) in which the original references can be found.

concentrations vastly to exceed the corresponding K_M's. Actually, the effective K_M's of many metabolites *in vivo* are much higher than the values given in Table II, because there is competition of substrates and products at the active sites of the different enzymes. Thus, the apparent K_M/S ratios of the metabolites of, for example, PGI, ALD, TIM, and GAPDH are substantially higher than the values given in the table. Furthermore, there must be an enormous sequestration of some metabolites because of the high concentrations of active sites inside the glycosome. The most obvious example is GA-3-P, which is present at a concentration equal to the active site concentration of GAPDH (0.5 mM).

CONTROL ANALYSIS

Because of the vital role of glycolysis in the trypanosomes' free-energy supply, and the fact that its organization in these parasites differs significantly from that in their host, this pathway is considered a main target of drug design (Michels, 1988). To inhibit specifically glycolysis of the parasite, classically one should choose either a unique step or the rate-limiting step of the pathway. The GPO reaction is such a unique step. It is, however, not an essential step, because under anaerobic conditions glycolysis proceeds at the same rate (Visser, 1981). Concerning the rate-limiting step, Metabolic Control Analysis (for reviews, see Westerhoff and Van Dam, 1987; Fell, 1992) has shown that there is in general not one rate-limiting step, but that the control of a steady-state flux may be distributed. Quantitatively this can be expressed in terms of the flux control coefficients $C^J_{e_i} = (dJ/J)/(de_i/e_i) = (d \ln J)/(d \ln e_i)$, where $C^J_{e_i}$ is the flux control coefficient of enzyme i on the flux J and e_i is the V_{max} of enzyme i. Similarly, one may define control coefficients to quantify the control of the flux by other parameters (Burns *et al.*, 1985). The sum of the flux control coeffi-

cients over all enzymes in the pathway is 1. Furthermore, the direct effect of metabolite concentrations on individual reaction rates is quantified by the elasticity coefficients $\epsilon^{v_i}_M = \partial v_i/v_i)/(\partial[M]/[M])$, where [M] is a metabolite concentration, v_i is the rate of enzyme i and ∂ indicates that these derivatives are partial derivatives, at fixed values of all other properties that affect the enzyme.

The control coefficients are related to the elasticity coefficients by connectivity theorems, such as $\sum_i C^J_{e_i} \cdot \epsilon^{v_i}_M = 0$, where the sum is over all enzymes in the pathway and [M] can be any metabolite concentration.

The distribution of control may depend on the conditions and may differ from one organism to another. Control Analysis can be helpful for drug design, because inhibiting a step that has a high control on the glycolytic flux in trypanosomes, but not in the host, should enhance drug selectivity. More fundamentally, it is interesting to investigate whether compartmentation and its consequences for regulation affect the control of glycolysis.

To measure flux control coefficients directly one should be able to vary gradually and exclusively the activity of one enzyme in the pathway. Until now this has not been accomplished for any of the glycolytic enzymes in trypanosomes. There are, however, data available that give us some clues about the distribution of control. We will distinguish between anaerobic and aerobic conditions.

CONTROL BY EXTERNAL PARAMETERS

In the bloodstream, trypanosomes encounter constant temperature, glucose concentration, and pH, but a strongly varying oxygen tension. The glucose concentration in blood is approximately 5 mM, which is far above the apparent K_M of 0.49 mM of the glycolytic flux for glucose, as determined in intact trypanosomes (Seyfang and Duszenko, 1991). The oxygen tension in blood varies between 13 kPa in alveoli of lungs and 3 kPa in active muscles, while the oxygen consumption rate is decreasing only below 1 kPa. Therefore, the glucose concentration and the oxygen tension are usually not controlling the glycolytic rate.

Nevertheless, trypanosomes living in the extracellular body fluids may encounter conditions where the oxygen supply becomes limited. They must switch then from production of two molecules of pyruvate per molecule of glucose to one pyruvate plus one glycerol per glucose. Indeed Eisenthal and Panes (1985) observed a steep increase of the ratio of production of glycerol and pyruvate when the oxygen tension dropped below 1 kPa. Concomitantly, the production of ATP drops from two to one molecule per molecule of glucose (cf. Fig. 2). As the rate of glucose consumption remains the same upon a switch from aerobic to anaerobic conditions (Visser, 1981), this suggests that the rate of ATP production may drop twofold. It is, therefore, the rate of ATP production rather than the rate of glucose consumption that may be controlled by the availability of oxygen.

AEROBIC CONDITIONS

The uptake of glucose has been proposed to be the rate-limiting step of glycolysis in trypanosomes (Gruenberg *et al.*, 1978; Ter Kuile and Opperdoes, 1991; Seyfang and Duszenko, 1991). The term "rate-limiting step" lacks the subtlety required for proper analysis of control and regulation. Therefore, we shall turn to the magnitude of the flux control coefficient of the glucose translocator. We translate the proposal of these authors into the statement that glucose transport has a control coefficient of 1, such that the control coefficients of all glycolytic enzymes must be zero, if we disregard the possibility of negative flux control coefficients.

Actually, glucose is transported both across the plasma membrane and across the glycosomal membrane. Until now no distinction between these two transport steps has been made in kinetic measurements. Uptake of glucose occurs via a facilitated diffusion carrier (Eisenthal *et al.*, 1989; Ter Kuile and Opperdoes, 1991). The carrier has a K_M of 2 mM for external D-glucose (Ter Kuile and Opperdoes, 1991) and, characteristic of facilitated diffusion carriers, it exhibits transacceleration, i.e., the V_{max} of uptake of radiolabeled glucose is twice as high in an equilibrium exchange experiment compared to a zero-trans influx or efflux experiment (Eisenthal *et al.*, 1989).

The hypothesis that hexose transport has a flux control coefficient of 1 is based on several lines of argumentation. First, D-glucose, D-fructose, and D-mannose are metabolized at different rates, suggesting that either transport or phosphorylation of sugars has some control (Gruenberg *et al.*, 1978). Secondly, the V_{max} of the carrier is 5.9 nmol $(10^8$ cells$)^{-1}$ $(7.5$ s$)^{-1}$ (Ter Kuile and Opperdoes, 1991), equivalent to 0.1

μmol (mg cell protein)$^{-1}$ min^{-1}, which is comparable to the rate of oxygen consumption: 0.1 μmol (mg cell protein)$^{-1}$ min^{-1}. A calculation factor of 1 mg protein per $2.25 \cdot 10^8$ cells was used (Seyfang and Duszenko, 1991). Thirdly, Ter Kuile and Opperdoes (1991) measured the internal glucose concentration at 5 mM of glucose outside and it remained as low as 0.4 mM. The authors took this as evidence that glucose transport is rate limiting. However, not every reaction that is far from equilibrium has a high flux control coefficient. Control coefficients are determined by the relative values of the elasticity coefficients, rather than by the distance from equilibrium (Kacser and Burns, 1973). To calculate the elasticity coefficient of the glucose carrier for the intracellular glucose concentration, we assume that this carrier is symmetric and works according to a 4-state model (Stein, 1986). The V_{max}'s and K_M's measured in trans-zero influx and efflux experiments actually differ by a factor of 1.5 approximately, but as the V_{max} for efflux is lower, while the K_M is higher compared to an influx experiment (Eisenthal *et al.*, 1989), this would violate the Haldane relation. When using a K_M of 2 mM, as measured in influx experiments, and a V_{max} for equilibrium exchange that is twice as high as for zero-trans influx (Eisenthal *et al.*, 1989) the rate equation becomes

$$v = \frac{V_{12}K(G_0 - G_i)}{(1.5K^2 + KG_0 + KG_i + 0.5G_0 G_i)}$$

with $K = 1.3$ mM, V_{12} is the V_{max} measured in a zero-trans influx experiment, and G_0 and G_i are the external and internal glucose concentration, respectively. The elasticity coefficient of the glucose transport rate for G_i is then

$$\epsilon_{G_i}^{\text{transport}} = \frac{\partial v/v}{\partial G_i/G_i} = -0.2$$

at $G_0 = 5$ mM and $G_i = 0.4$ mM. As the K_M of HK for glucose has been measured to be 0.1 mM (A. Loiseau and F. R. Opperdoes, personal communication) the elasticity coefficient of HK for G_i may be estimated as

$$\epsilon_{G_i}^{\text{HK}} = \frac{\partial v_{\text{HK}}/v_{\text{HK}}}{\partial G_i/G_i} = \frac{K_M}{K_M + G_i} = 0.2$$

Assuming that other reaction rates are independent of G_i, the connectivity theorem for the flux and G_i is

$$C^J_{\text{transport}} \cdot \epsilon_{G_i}^{\text{transport}} + C^J_{\text{HK}} \cdot \epsilon_{G_i}^{\text{HK}} = 0$$

It follows that $C^J_{\text{transport}}/C^J_{\text{HK}} = -\epsilon_{G_i}^{\text{HK}}/\epsilon_{G_i}^{\text{transport}} = 1$. Because it is unlikely that there is a strongly negative flux control by an enzyme in the system, this suggests that the transport step has a control coefficient lower that 0.5. With a reversible Michaelis–Menten equation for the transport step, this conclusion remains qualitatively the same. The above calculation is based on the assumption that there is only a single intracellular glucose pool. Although no definitive conclusion should be drawn from these calculations, they should serve to demonstrate that a low internal glucose concentration in itself is insufficient to conclude that glucose transport has a flux control coefficient of 1.

There are more indications that glucose transport has a flux control coefficient lower than 1. First, the inhibition of the glucose carrier by phloretin (Seyfang and Duszenko, 1993) is much stronger than the inhibition of the flux by this inhibitor (Seyfang and Duszenko, 1991). Secondly, the K_M of the glucose carrier for glucose is 2 mM, while the apparent K_M of the steady-state flux for glucose is 0.49 mM. If the carrier had a control coefficient of 1, one would expect that the apparent K_M of the flux for glucose were at least as high as the K_M of the carrier itself. Our own inhibitor titrations confirm that the flux control coefficient of glucose transport is significant, but less than 1 (B. M. Bakker, P. A. M. Michels, F. R. Opperdoes, and H. V. Westerhoff, unpublished data).

The summation theorem for flux control coefficients demonstrates that the sum of the flux control coefficients in a pathway is equal to 1. As we propose that the flux control coefficient of the glucose carrier is significant, but lower than 1, this leaves the possibility for other steps in glycolysis to have some control.

A second step that possibly exerts some control on the glycolytic flux is the glycosomal GAPDH. *T. brucei* has two GAPDH isoenzymes, of which one is situated in the cytosol and the other is imported into the glycosome (Misset *et al.*, 1987; Lambeir *et al.*, 1991). Both the cytosolic and the glycosomal GAPDH are inhibited by 3-bromopyruvate, which binds covalently to the active site cysteine of the enzyme. Barnard *et al.* (1993) showed that 3 μM of 3-bromopyruvate caused a 50% inhibition of the total GAPDH activity *in vivo*, while 6 μM was required to inhibit the glycolytic flux by 50%. From amino-terminal sequencing of a protein binding to [^{14}C]3-bromopyruvate, it turned out that the inhibitor reacts preferentially with the glycosomal GAPDH isoenzyme. The inhibition of glycolysis cannot be explained by inhibition of the pyruvate transporter or PYK, because these proteins have a K_i above 100 μM for 3-bromopyruvate. It can, however, not be excluded that other cysteine-containing

enzymes or transporters are also inhibited by 3-bromopyruvate.

By inhibitor titrations we determined that the pyruvate transporter in the plasma membrane, which is also a facilitated diffusion carrier (Wiemer *et al.*, 1992; Barnard *et al.*, 1993), has a small but significant flux control coefficient (B. M. Bakker, P. A. M. Michels, F. R. Opperdoes, and H. V. Westerhoff, unpublished data).

When investigating the control of a metabolic pathway, one should in general not only look at the reactions of that pathway itself, but also at the reactions that are linked to it. As the production of ATP is the main function of glycolysis in bloodstream form trypanosomes, ATP-utilizing processes may also control glycolysis. The main ATP-utilizing processes are growth, motility, and possibly the maintenance of electrochemical potential differences across the mitochondrial membrane and the plasma membrane.

It has been demonstrated that oligomycin, which inhibits the mitochondrial H^+-ATPase, also inhibits the rates of oxygen consumption and pyruvate production, with a concomitant dissipation of the electric potentials across the mitochondrial membrane and the plasma membrane, when glucose is the substrate (Nolan and Voorheis, 1992; Kiaira and Njogu, 1994). One explanation could be that inhibition of ATP utilization by the mitochondrial H^+-ATPase causes a lack of cytosolic ADP for the PYK reaction, thereby inhibiting glycolysis. The [ATP]/[ADP] ratio decreased rather than increased, however, upon incubation with oligomycin (Nolan and Voorheis, 1992; Kiaira and Njogu, 1994). If there were proton pumping by GPO and thus, mitochondrial ATP production, this could explain the drop of the [ATP]/[ADP] ratio upon addition of oligomycin, but not the dissipation of the mitochondrial membrane potential. Furthermore, with glycerol as a catabolic substrate, the pyruvate production and oxygen consumption rates are insensitive to oligomycin, which does not fit with either explanation. Direct inhibition of transport or phosphorylation of glucose by oligomycin could not be demonstrated (Kiaira and Njogu, 1994). These experiments were all carried out with nongrowing cell suspensions. Even if the glycolytic flux is controlled partially by ATP-utilizing reactions in such suspensions, these reactions may lack control in growing cells, because growth requires more ATP.

ANAEROBIC CONDITIONS

Under anaerobic conditions, bloodstream form *T. brucei* produces equimolar amounts of pyruvate and glycerol (Visser, 1981). A very interesting step under those conditions is the reaction catalyzed by GK: the conversion of G-3-P into glycerol, with the concomitant production of ATP. This reaction has a strongly positive $\Delta G_0'$ (+22 kJ/mol) and, therefore, very high concentrations of the substrates G-3-P and ADP are required. Indeed, the concentration of G-3-P increases 4-fold, from 0.9 to 3.6 μmol/g wet weight, upon transition from aerobic to anaerobic conditions (Visser and Opperdoes, 1980). These numbers reflect the total G-3-P concentration in the cell. The glycosomal concentrations of this intermediate and ADP may well increase more dramatically. Furthermore, the glycolytic flux is strongly and immediately inhibited by glycerol in the presence of salicylhydroxamic acid (Fairlamb *et al.*, 1977). Therefore, it is very probable that the GK reaction has some control under anaerobic conditions.

CONTROL OF GLYCOLYSIS IN OTHER EUKARYOTES

In order to relate the control of trypanosomal glycolysis to the special organization of this pathway in the parasite, we will compare this control to the control of glycolysis in yeast and mammals. Several studies on control of glycolysis have been published. Until now, however, hardly anybody varied enzyme activities near wild type level, because suitable inhibitors and tunable expression systems around wild type levels have been lacking.

Some studies were performed with cell extracts, which has the advantage that purified enzymes can be added. With this method one neglects that substantial control can be exerted by transport steps and, as the flux control coefficients always sum up to 1, one overestimates the other control coefficients. An alternative is overexpression of glycolytic enzymes. Often one makes use of transfected cells that overproduce enzymes manifold. Control can shift then to other enzymes, so that this method underestimates control coefficients. Furthermore, the expression levels of other enzymes can also change in such genetically modified cells. A technically very complicated method is inversion of the elasticity matrix to calculate control coefficients. One measures then fluxes and metabolite concentrations and uses a rate equation for each step to calculate the elasticity coefficients. This method has two main problems. In the first place, it only works if one looks at a complete pathway, including all branches connected to this pathway. This is in practice

not always feasible. Furthermore, this method heavily relies on the rate equations used.

YEAST

Not only does the glycolytic pathway of the yeast *Saccharomyces cerevisiae* differ from that of trypanosomes, but also the conditions in which this yeast lives are very different. While bloodstream form trypanosomes are optimized for a life under constant conditions, *S. cerevisiae* has to be capable of quickly adapting to changing conditions. As *S. cerevisiae* grows almost completely fermentatively when glucose is available in excess, a comparison with *T. brucei* is appropriate. An important difference between *S. cerevisiae* and bloodstream form *T. brucei* is that the former accumulates a substantial amount of glycogen and trehalose, while the latter does not.

As the availability of specific, *in vivo* acting inhibitors of glycolytic enzymes is limited, most studies aiming to measure flux control coefficients of glycolytic enzymes in yeast made use of overexpression of these enzymes. Effects of overexpression on both the glycolytic flux and the growth rate have been studied. Schaaff *et al.* (1989) showed that a manifold overexpression of most glycolytic enzymes, including HK (14-fold), PFK (4-fold), and PYK (9-fold), hardly increased or even decreased the glycolytic flux and the growth rate. Overexpression of PGI (11-fold) (Benevolensky *et al.*, 1994) and phosphoglycerate kinase (PGK, 4- to 28-fold) (Brindle, 1988) did not significantly affect the flux either. Concomitantly with a 4-fold overexpression of PFK, the activity of 6-phosphofructo-2-kinase and the concentration of fructose 2,6-bisphosphate decreased by a factor of 2 under anaerobic conditions, thus decreasing the *in vivo* activity of PFK (Davies and Brindle, 1992). This explains why PFK does not have any control on the glycolytic flux in growing cells. Whether PFK has any control at the metabolic level, i.e., if the activities of other enzymes are kept constant, remains to be established. As the glycosomal PFK activity in *T. brucei* is not regulated by fructose 2,6-bisphosphate, it should be interesting to investigate whether PFK does have control in this organism. For the other enzymes overexpressed in yeast, it is not clear whether they really do not have any control on the flux or whether the concentrations of other enzymes are decreased aspecifically, because of the enormous overexpression of one enzyme. Indeed, Snoep *et al.* (personal communication) showed that overexpression of one enzyme in the bacterium *Zymomonas mobilis* caused a proportional decrease of other enzyme activities. Titration of GAPDH did not result in a change of the flux either (Brindle, 1988).

If indeed the glycolytic enzymes themselves do not exert any control, then it is possible that the control resides in the glucose transport system or in ATP-utilizing reactions. Galazzo *et al.* (1990) made a kinetic model of anaerobic glycolysis in yeast to calculate the flux control coefficients of some enzymes from the elasticity coefficients, using the metabolite concentrations they measured. They reported flux control coefficients of 0.45 for glucose transport, 0.20 for PFK, and 0.35 for ATPase at pH 5.5. When the pH was decreased, thereby activating ATPase, control shifted from ATPase to glucose transport. The rate equations these authors used for glucose uptake and ATP utilization are rather speculative, though. The control calculated for the transport system depends strongly on the strength of a supposed inhibition of the transport system by hexosephosphates. This effect itself is ill documented. Furthermore, the authors did not include NAD^+ and NADH in their calculations.

MAMMALIAN CELLS

One cannot expect to find that the control of glycolysis is the same in all mammalian cells. Different tissues have each their specific function in the body and, as a consequence, differ in their overall metabolic pattern. The importance of glycolysis may vary from one cell type to another, and it can also be influenced by developmental, hormonal, or nutritional factors. Furthermore, the variations in glycolysis may involve different isoenzymes, each with their specific kinetic properties and mechanisms of activity regulation (Fothergill-Gilmore and Michels, 1993).

So far, only a limited number of studies have been reported which provide information about the flux control coefficients in mammalian cells. Rapoport *et al.* (1976) doubled the activity of HK, PFK, GAPDH, and PYK in human erythrocyte lysates by addition of purified enzymes and determined the effect on glucose consumption and lactate production. Only HK and PFK were found to exert a positive flux control. The flux increased about 30 and 40%, respectively, upon addition of those enzymes. Major control by those enzymes in red blood cells was consistent with the predictions from a kinetic model (Rapoport *et al.*,

1974, 1976). Control of glycolysis in human erythrocytes by HK was confirmed in an analysis by Magnani *et al.* (1988). Cells were loaded with exogenous enzyme, by hemolysis and resealing. A 15-fold increase in HK activity resulted in a doubling of lactate production. Using *in vitro* mammalian model systems, Torres *et al.* (1986, 1989, 1990) studied control in a shortened glycolytic pathway, comprising the six enzymes responsible for the conversion of glucose into G-3-P. Control appeared to be distributed over HK and PFK. However, the latter experiments bear limited relevance to control of glycolysis *in vivo,* because of the truncated pathway studied.

All aforementioned studies focused on only a few enzymes of the pathway and were in most cases performed with cell lysates, so that transport steps were excluded from the analyses. A comprehensive study, applying the matrix inversion method, was recently reported about glucose utilization, also including glycogen synthesis, in perfused rat heart (Kashiwaya *et al.*, 1994). This paper described that control in heart cells is variably distributed among several enzymes, depending upon substrate availability, hormonal stimulation, and other changes of conditions. PFK was not included, however, and the GAPDH reaction was assumed to be in equilibrium. In the absence of insulin most control within the glycolytic branch was shared by the glucose transporter in the plasma membrane and HK; the ratio $C^J_{\text{transport}}/C^J_{\text{HK}}$ varied between 0.5 and 0.7. The analysis confirmed the conjecture that control by the glucose transport step was abolished in the presence of insulin.

CONCLUSIONS AND REMAINING QUESTIONS

In the above sections, we discussed the organization, regulation, and control of compartmentalized glycolysis in bloodstream form trypanosomes and we made a comparison with other cell types where glycolysis takes place in the cytosol.

The insensitivity of the glycosomal enzymes to many regulatory compounds is compatible with the compartmentation of the pathway. As the glycosomal enzymes are not in direct contact with the cytosolic [ATP]/[ADP] ratio and this ratio is probably very different in the glycosomes, there is no reason for regulation of glycosomal enzymes by [ATP]/[ADP]. Furthermore, as gluconeogenesis, if present, is localized in the cytosol, regulation of PFK by fructose 2,6-bisphosphate, to prevent futile cycling, seems unnecessary. The precise regulatory function of the activation of the trypanosomal PYK by fructose 2,6-bisphosphate is not clear yet. The compartmentation of glycolysis also leads to a special moiety-conserved sum that comprises all metabolites in the glycosome possessing a transferable phosphate group.

Whether and how compartmentation of glycolysis has an advantage for cell functioning, remains an intriguing but unsolved question. We could not find any evidence for the hypothesis that compartmentation enables the high glycolytic flux by overcoming a diffusion limitation. If diffusion is not especially hindered, the amounts of glycolytic enzymes are so high, that diffusion should not be limiting the flux, even if the enzymes were not compartmentalized. The high concentrations of metabolites in the glycosome, which had been related to a possible diffusion limitation, may be due to sequestration by the enzymes that are present in very high concentrations.

Whether the special organization of glycolysis in trypanosomes results in a different control of the pathway is also unclear yet, although answers are forthcoming. In trypanosomes living in their mammalian host, the rate of glycolytic ATP production is not controlled by the glucose concentration, which is in excess, but sometimes by the oxygen tension, because the cells shift to a less efficient route when the oxygen tension becomes low. Furthermore, we argued that glucose transport is not likely to be *the* rate-limiting step of glycolysis in trypanosomes, as was suggested before. Probably this transport step does have substantial control, but its control coefficient is lower than 1. In this respect, trypanosomes do not differ from yeast and mammalian cells, where the uptake of glucose also seems to exert some control on the flux, although the evidence is rather indirect. If glucose transport has a flux control coefficient lower than 1, other steps can also have some control. Under anaerobic conditions GK is a good candidate to have some control in trypanosomes, because it has a strongly positive $\Delta G_0'$. Under aerobic conditions, GAPDH and ATP-utilizing reactions are interesting candidates, but the available evidence is incomplete and for the latter also confusing. In yeast a relation could be established between regulation of PFK by fructose 2,6-bisphosphate and the fact that PFK overexpression hardly affects the glycolytic flux. Therefore, it will be interesting to determine whether the absence of such regulation of PFK in *T. brucei* would endow this enzyme with a higher flux control coefficient. To further investigate

control of glycolysis in trypanosomes, some questions concerning the structure of the pathway must be clarified. First, it remains to be established unambiguously whether GPO is translocating protons across the mitochondrial membrane and thus, whether there is mitochondrial ATP synthesis. Secondly, specific transporters of metabolites across the glycosomal membrane have not been identified until now. We are especially interested in a possibility to modulate the transport of glucose across the glycosomal membrane as we argue that glucose transport exerts some control on the flux, but one cannot distinguish between control by the transport across the plasma membrane and the glycosomal membrane yet. Furthermore, it will be important to determine the concentration of glucose and the [ATP]/[ADP] ratio within the organelle.

Most importantly, the conclusion that some but not all control of the flux resides in glucose transport calls for quantitative assessments of the distribution of control. To quantitatively measure flux control coefficients, it will be necessary to vary the activity of the glycolytic enzymes independently, near wild type level. Then it will become feasible to investigate whether there is a relation between compartmentation and the distribution of control of glycolysis.

ACKNOWLEDGMENTS

The authors would like to thank B. Teusink and Drs. F. R. Opperdoes and L. Hue for fruitful discussions and careful reading of the manuscript and Ms. N. Chevalier for the excellent drawing of Fig. 2. This study was supported by the Netherlands Organization for Scientific Research (NWO) and the "Fonds de la Recherche Scientifique Medicale" (Belgium).

REFERENCES

Aman, R. A., Kenyon, G. L., and Wang, C. C. (1985). *J. Biol. Chem.* **260**, 6966–6973.
Barnard, J. P., Reynafarje, B., and Pedersen, P. L. (1993). *J. Biol. Chem.* **268**, 3654–3661.
Benevolensky, S. V., Clifton, D., and Fraenkel, D. G. (1994). *J. Biol. Chem.* **7**, 4878–4882.
Bienen, E. J., Saric, M., Pollakis, G., Grady, R. W., and Clarkson, A. B., Jr. (1991). *Mol. Biochem. Parasitol.* **45**, 185–192.
Brindle, K. M. (1988). *Biochemistry* **27**, 6187–6196.
Burns, J. A., Cornish-Bowden, A., Groen, A. K., Heinrich, R., Kacser, H., Porteous, J. W., Rapoport, S. M., Rapoport, T. A., Stucki, J. W., Tager, J. M., Wanders, R. J. A., and Westerhoff, H. V. (1985). *Trends Biochem. Sci.* **10**, 1–6.
Callens, M., Kuntz, D. A., and Opperdoes, F. R. (1991a). *Mol. Biochem. Parasitol.* **47**, 19–30.
Callens, M., Kuntz, D. A., and Opperdoes, F. R. (1991b). *Mol. Biochem. Parasitol.* **47**, 1–10.
Callens, M., and Opperdoes, F. R. (1992). *Mol. Biochem. Parasitol.* **50**, 235–244.
Clarkson, A. B., Jr., Bienen, E. J., Pollakis, G., and Grady, R. W. (1989). *J. Biol. Chem.* **264**, 17770–17776.
Clegg, J. S. (1986). In *The Organization of Cell Metabolism* (Welch, G. R., and Clegg, J. S., eds.), Plenum Press, New York, pp. 41–56.
Cronin, C. N., and Tipton, K. F. (1985). *Biochem. J.* **227**, 113–124.
Cronin, C. N., and Tipton, K. F. (1987). *Biochem. J.* **245**, 13–18.
Cronin, C. N., Nolan, D. P., and Voorheis, H. P. (1989). *FEBS Lett.* **244**, 26–30.
Davies, S. E. C., and Brindle, K. M. (1992). *Biochemistry* **31**, 4729–4735.
Eisenthal, R., and Panes, A. (1985). *FEBS Lett.* **181**, 23–27.
Eisenthal, R., Game, S., and Holman, G. D. (1989). *Biochim. Biophys. Acta* **985**, 81–89.
Fairlamb, A. H., Opperdoes, F. R., and Borst, P. (1977). *Nature* **265**, 270–271.
Fell, D. A. (1992). *Biochem. J.* **286**, 313–330.
Fersht, A. (1985). *Enzyme Structure and Mechanism,* 2nd edn., W. H. Freeman, New York.
Fothergill-Gilmore, L. A., and Michels, P. A. M. (1993). *Prog. Biophys. Mol. Biol.* **59**, 105–235.
Galazzo, J. L., and Bailey, J. E. (1990). *Enzyme Microb. Technol.* **12**, 162–172. Errata, **13**, 363.
Grant, P. T., and Sargent, J. R. (1960). *Biochem. J.* **76**, 229–237.
Gruenberg, J., Sharma, P. R., and Deshusses, J. (1978). *Eur. J. Biochem.* **89**, 461–469.
Hammond, D. J., and Bowman, I. B. R. (1980). *Mol. Biochem. Parasitol.* **2**, 77–91.
Hammond, D. J., Aman, R. A., and Wang, C. C. (1985). *J. Biol. Chem.* **29**, 15646–15654.
Hannaert, V., and Michels, P. A. M. (1994). *J. Bioenerg. Biomembr.* **26**, 205–212.
Kacser, H., and Burns, J. A. (1973). In *Rate Control of Biological Processes* (Davies, D. D., ed.), Cambridge University Press, London, pp. 65–109.
Kashiwaya, Y., Sato, K., Tsuchiya, N., Thomas, S., Fell, D. A., Veech, R. L., and Passonneau, J. V. (1994). *J. Biol. Chem.* **269**, 25502–25514.
Kiaira, J. K., and Njogu, M. R. (1994). *Biotechnol. Appl. Biochem.* **20**, 347–356.
Lambeir, A.-M., Opperdoes, F. R., and Wierenga, R. K. (1987). *Eur. J. Biochem.* **168**, 69–74.
Lambeir, A.-M., Loiseau, A. M., Kuntz, D. A., Vellieux, F. M., Michels, P. A. M., and Opperdoes, F. R. (1991). *Eur. J. Biochem.* **198**, 429–435.
Magnani, M., Rossi, L., Bianchi, M., Fornaini, G., Benatti, U., Guida, L., Zocchi, E., and De Flora, A. (1988). *Biochim. Biophys. Acta* **972**, 1–8.
Marchand, M., Kooystra, U., Wieringa, R. K., Lambeir, A.-M., Van Beeumen, J., Opperdoes, F. R., and Michels, P. A. M. (1989). *Eur. J. Biochem.* **184**, 455–464.
Michels, P. A. M. (1988). *Biology of the Cell* **64**, 157–164.
Michels, P. A. M., and Hannaert, V. (1994). *J. Bioenerg. Biomembr.* **26**, 213–219.
Misset, O., and Opperdoes, F. R. (1984). *Eur. J. Biochem.* **144**, 475–483.
Misset, O., Bos, O. J. M., and Opperdoes, F. R. (1986). *Eur. J. Biochem.* **157**, 441–453.
Misset, O., Van Beeumen, J., Lambeir, A.-M., Van der Meer, R., and Opperdoes, F. R. (1987). *Eur. J. Biochem.* **162**, 501–507.
Nolan, D. P., and Voorheis, H. P. (1992). *Eur. J. Biochem.* **209**, 207–216.

Nwagwu, M., and Opperdoes, F. R. (1982). *Acta Tropica* **39**, 61–72.
Opperdoes, F. R. (1987). *Annu. Rev. Microbiol.* **41**, 127–151.
Opperdoes, F. R., and Borst, P. (1977). *FEBS Lett.* **80**, 360–364.
Rapoport, T. A., Heinrich, R., Jacobasch, G., and Rapoport, S. (1974). *Eur. J. Biochem.* **42**, 107–120.
Rapoport, T. A., Heinrich, R., and Rapoport, S. (1976). *Biochem. J.* **154**, 449–469.
Schaaff, I. Heinisch, J., and Zimmermann, F. K. (1989). *Yeast* **5**, 285–290.
Seyfang, A., and Duszenko, M. (1991). *Eur. J. Biochem.* **202**, 191–196.
Seyfang, A., and Duszenko, M. (1993). *Eur. J. Biochem.* **214**, 593–597.
Shapiro, T. A., and Talalay, P. (1982). *Exp. Parasitol.* **54**, 379–390.
Stein, W. D. (1986). *Transport and Diffusion across Cell Membranes.* Academic Press, London.
Ter Kuile, B. H., and Opperdoes, F. R. (1991). *J. Biol. Chem.* **266**, 857–862.
Torres, N. V., Mateo, F., Melendez-Hevia, and Kacser, H. (1986). *Biochem. J.* **234**, 169–174.
Torres, N. V., Souto, R., and Melendez-Hevia, E. (1989). *Biochem. J.* **260**, 763–769.
Torres, N. V., Mateo, F., Riol-Cimas, J. M., and Melendez-Hevia, E. (1990). *Mol. Cell. Biochem.* **93**, 21–26.
Van Schaftingen, E., Opperdoes, F. R., and Hers, H.-G. (1985). *Eur. J. Biochem.* **153**, 403–406.
Van Schaftingen, E., Opperdoes, F. R., and Hers, H.-G. (1987). *Eur. J. Biochem.* **166**, 653–661.
Visser, N., and Opperdoes, F. R. (1980). *Eur. J. Biochem.* **103**, 623–632.
Visser, N., Opperdoes, F. R., and Borst, P. (1981). *Eur. J. Biochem.* **118**, 521–526.
Visser, N. (1981). *Carbohydrate Metabolism in Erythrocytes and Trypanosomes,* PhD thesis, University of Amsterdam.
Westerhoff, H. V. (1985). *Nature* **318**, 106.
Westerhoff, H. V. and Van Dam, K. (1987). *Thermodynamics and Control of Biological Free-Energy Transduction,* Elsevier, Amsterdam.
Westerhoff, H. V., and Welch, G. R. (1992). *Curr. Top. Cell. Regul.* **33**, 361–390.
Wiemer, E. A., Ter Kuile, B. H., Michels, P. A. M., and Opperdoes, F. R. (1992). *Biochem. Biophys. Res. Commun.* **184**, 1028–1034.