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Theoretical approaches to the evolutionary optimization of glycolysis
Chemical analysis

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In the first part of this work [Heinrich, R., Montero, F., Klipp, E., Waddell, T. G. & Meléndez-Hevia, E. (1997) *Eur. J. Biochem. 243*, 191–201] the kinetic and thermodynamic constraints under which an optimal glycolysis must be designed have been analysed. In this second part, we present a chemical analysis of the glycolytic pathway in order to determine if its design is chemically optimized according the possibilities that a glycolytic design can have. Our results demonstrate that glycolysis in modern-day cells (from glucose to lactate) has an optimized design for maximizing the flux of ATP production, and a thermodynamic profile which guarantees a high kinetic efficiency. We also discuss some cases of paleometabolism for this pathway as alternative metabolic pathways, less optimized, that exist in some bacteria. Our analysis relates mainly to metabolism designed under constant chemical affinity (substrates and products of the pathway constant), where the target of optimization can be the flux of ATP production. We also discuss the case of an externally imposed input flux, whose target of optimization is the stoichiometric yield of ATP.

Keywords: glycolysis; optimization; evolution; metabolism; metabolic design.

It is now well recognized that natural selection is an especially successful mechanism of optimization which operates in biological evolution. However, the number of cases where it has been well documented is not large. The reason for this is that any biological problem usually has a large number of variables, so it is not easy to study or define an obvious function of optimization in order to derive a relationship among all the variables in a mathematically operative way. Cellular metabolism is a very interesting field in which to study biological evolution and natural selection, since a number of clear and interesting problems of optimization are involved and the number of variables is controllable, allowing an analytical solution in many cases.

An interesting subject in the evolution of metabolism is the strategy for achieving a good design of metabolic pathways, since rules of Chemistry can allow a number of different chemical means to solve a given metabolic problem, although with different effectiveness. In previous work, we have studied some cases of metabolic optimization, namely the design of the pentose phosphate cycle and the Calvin cycle in photosynthesis [1–5], the glycogen structure [6, 7], and the Krebs' cycle [8]. We have demonstrated that the simplest possible design accounts for the maximization of flux under constant values of other variables [4]; thus, we have concluded that the maximization of metabolic flux has been an important target in metabolic evolution for the fitness optimization variable. Simplicity in chemical design also accounts for minimization of the response time and minimization of the diversity of intermediate pools. Optimization of the glycogen structure is also in agreement with this conclusion.

In a different series of papers, we have also studied the optimization of kinetic constants of enzymes to achieve the maximum velocity (catalytic efficiency) [9, 10] and the kinetic design of enzymic chains of reactions [11, 12]. These are additional important aspects of metabolic evolution, closely related to the aim of the work presented here, since evolution also works on fine adjustment of each enzyme independently of establishing the sequence of steps. More aspects of metabolic optimization studied by other authors are available in [13].

In our previous research, we examined a kinetic objective, but no thermodynamic consideration was taken into account. In

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Abbreviations. Gro, glycerol; Grn, dihydroxyacetonephosphate; Gri, glyceric acid; Gra, glyceraldehyde; q_i, equilibrium constant; ΔG^c, standard cellular free energy.

Enzymes. Acyl-CoA synthetase (EC 6.2.1.3); aldolase (EC 4.1.2.13); amino-acid *N*-acetyltransferase (EC 2.3.1.1); creatine kinase (EC 2.7.3.2); enolase (EC 4.2.1.11); fumarate reductase (NADH) (EC 1.3.1.6); gluconokinase (EC 2.7.1.12); glyceraldehyde 3-*P* dehydrogenase (EC 1.2.1.12); hexokinase (EC 2.7.1.1); lactate dehydrogenase (EC 1.1.1.27); methylmalonyl-CoA mutase (EC 5.4.99.2); 6-phosphofructokinase (EC 2.7.1.11); phosphoglucomutase (EC 5.4.2.2); phosphoglycerate kinase (EC 2.7.2.3); phosphoketolase (EC 4.1.2.9); 3-phosphoshikimate 1-carboxyvinyltransferase (EC 2.5.1.19); phosphotransferase system (EC 2.7.3.9, EC 2.7.1.69); pyrophosphatase (EC 3.6.1.1); PPi-fructose 6-*P* phosphotransferase (EC 2.7.1.90); propionyl-CoA carboxylase (EC 6.4.1.3); propionate CoA-transferase (EC 2.8.3.1); pyruvate kinase (EC 2.7.1.40); *P*-pyruvate-carboxykinase (GTP) (EC 4.1.1.32); succinate dehydrogenase (EC 1.3.5.1); succinyl-CoA transferase (EC 2.8.3.2); succinyl-CoA synthetase (EC 6.2.1.4); triosephosphate isomerase (EC 5.3.1.1); glycogen phosphorylase (EC 2.4.1.1).

this work, we present a study on the optimization of glycolysis. Previously [14] we analysed the influence of thermodynamic and kinetic constraints on the kinetic yield of ATP flux. The aim of this second study is to investigate the problems of chemical design which could be involved in the optimization of glycolysis. Glycolysis is considered as an example of an anaerobic pathway aimed to produce ATP; the aim of its optimization is to maximize the ATP production and to minimize the response time when the metabolic system changes its steady state. Maximization of ATP production can occur in two ways; maximization of the stoichiometric ATP yield or the ATP flux. These targets are maximized under different constraints. The problem of optimization is complicated because a good solution depends on three *variables*:

(a) The global exergonism, which is closely dependent on the net ATP yield/glucose transformed; in effect, little ATP produced/glucose molecule means bad coupling, and thus little energy gain. However, this is kinetically good because the high exergonism promotes a high chemical affinity, giving a large flux; this effect has already been discussed [14], and is described by Eqn (1)

$$v = \frac{k_d \cdot E_i \cdot (S_{i-1} \cdot q_i - S_i)}{1 + q_i} \qquad (1)$$

where E_i and k_d are kinetic parameters of the enzyme-catalyzed reaction, S_{i-1} and S_i are the concentrations of the substrate and the product, respectively, and q_i is the equilibrium constant of the reaction. If many molecules of ATP are produced, it implies a good exploitation of the fuel, but very low global exergonism and a poor chemical affinity, and so a very poor ATP flux.

(b) The distribution of local exergonisms along the pathway. The thermodynamic analysis developed in [14] led to a surprising result relating kinetic and thermodynamic features; in a metabolic system under a given total affinity (a net exergonism), the different distributions of the local exergonisms produce great differences in the kinetic efficiency of the system. Thus, assuming a given net ATP yield, there are several ways in which it can be achieved. According to the conclusions of [14], the best design to achieve a large flux is to concentrate the highly exergonic reactions at the beginning of the pathway, and to put the less exergonic reactions at the end (Fig. 1). Thus, taking into account the chemical constraints, the steps of ATP usage (if they exist) should be as near the beginning of the route as possible, and all ATP-formation steps should be as near the end of the route as possible; whether chemical mechanisms allow a glycolytic design of this type has to be explored.

(c) Simplicity of the chemical design accounts for a greater flux of chemical transformation, as mentioned above.

These three variables are not strictly independent, since the value of each usually affects the chemical possibilities that allow diversity of the others. Here, the chemical analysis of this problem is presented, showing that a number of different chemical solutions can exist. Then, the best solution according to these principles of optimization is analysed. Our results demonstrate that the regular design of cellular glycolysis (Fig. 2) is optimized and, thus, it is another example which proves that natural selection, working at the molecular level, has solved a complicated problem of optimization.

Hypotheses of the model. In modern-day cells, glycolysis has a number of different functions. However, from the point of view of metabolic evolution, the first purpose of glycolysis had to be a pathway to produce ATP anaerobically at the substrate level. Thereafter, such a pathway has persisted in the present cells because of a number of obvious advantages which allow life under anaerobic conditions [15–17] and guarantee a fast and quick response for ATP supply [18, 19].

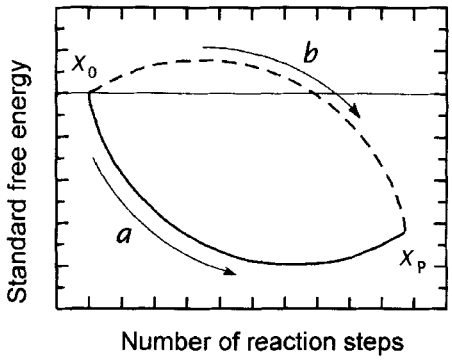


Fig. 1. Two different thermodynamic designs of a metabolic pathway. Two extreme (theoretical) thermodynamic profiles for the same whole conversion of X_0 into X_P. The exergonism ($-\Delta G°$) is the same for the two cases, but the conversion can be made through two different pathways, with the same number of steps, involving different local energy changes. (a) Design where the exergonic steps are placed at the beginning of the chain of reactions and the endergonic steps at the end. (b) Design with the most exergonic steps at the end of the pathway. The kinetic efficiency of each route is different according to the results described in the first part of this work [14]. (a) has an optimized thermodynamic design, while the design of (b) is poor. Therefore, under the same chemical affinity, and with the same kinetic properties (K_m and V_{max}) for each enzyme, the kinetic efficiency of (a) is much better than that of (b). The flux of conversion is much higher through (a) than through (b).

The anaerobic synthesis of ATP, considered as a chemical problem, has a number of different solutions, including the choice of the starting substrate and the end product. This problem can be considered in two parts; (a) the origin of glycolysis, as a carbon pathway which converts glucose into lactate, and (b) the optimization of its chemical design, to achieve the best sequence of chemical reactions. In this paper, the problem of optimization is studied, while the first question will be developed in a further work. The purpose of this paper is thus to analyze whether the sequence of chemical reactions in glycolysis has an optimized design according to the optimization criteria stated above. No chemical restriction for the reactions is imposed in this analysis other than those derived from thermodynamic and the general bioorganic mechanisms, which make a reaction possible with reasonably high equilibrium constant. According to these assumptions, our model is stated under the following hypotheses.

(a) Anaerobiosis. The whole process cannot depend on any external electron source or sink; redox reactions can of course occur but, in the global process, the initial substrate must have the same global oxidation degree as the end product.

(b) Exergonism. On the basis of a given reasonable set of external conditions, the global process of carbon conversion must have enough intrinsic exergonism to permit a positive balance of ATP production and also to make the whole conversion possible.

(c) Phosphorylation at the substrate level. In carbon conversion, it must be possible chemically to couple some steps of the conversion to the synthesis of ATP. This coupling must be at the substrate level because its mechanism must be strictly dependent only on the chain of 'glycolytic' reactions. Thermodynamic and chemical conditions for this mechanism are explained below.

(d) Polarity. Any intermediary metabolite must have a high polarity level which prohibits its free diffusion (escape) through the cellular membrane. This leads directly to the assumption that the first metabolic reaction on a sugar must give it a high degree of polarity, since free sugars have low polarity. This reaction is carried out in modern-day metabolism by means of ATP expedi-

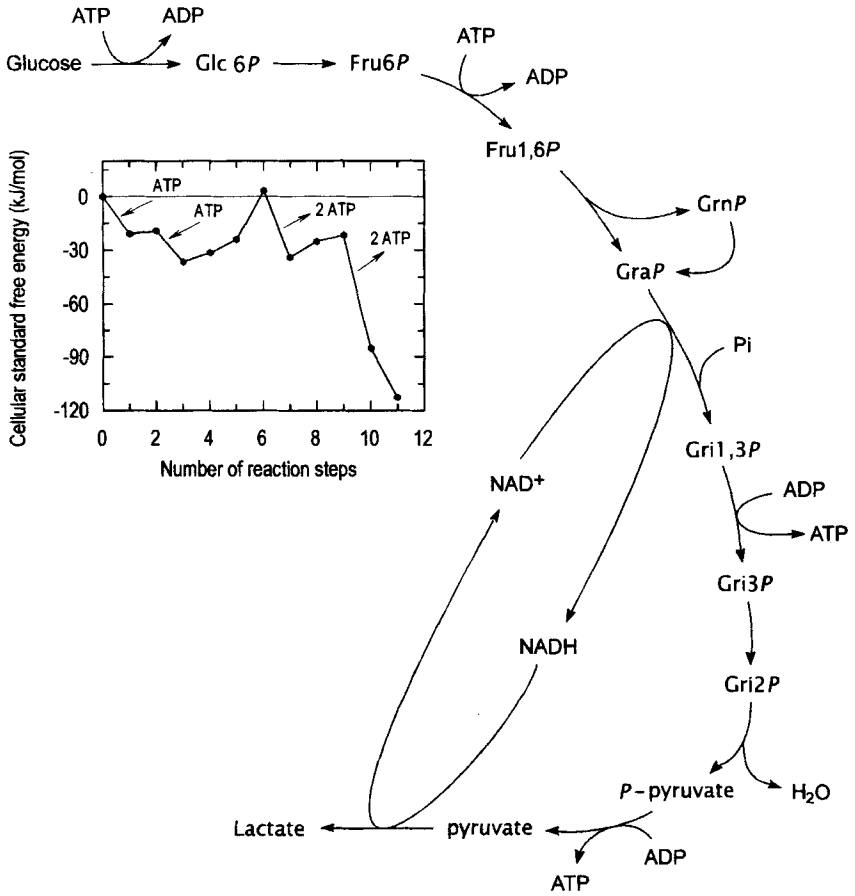


Fig. 2. The standard glycolytic pathway. Design of standard glycolysis, as occurs in most modern cells. This is the most optimized design of a pathway to maximize the flux of ATP production under anaerobic conditions (all redox reactions balanced). The thermodynamic profile is shown in the inset as ΔG^c.

ture. Thus, sugar metabolism in the present cells is organized around phosphorylated sugars. Phosphorylation is not necessary if the first reaction is an alternative means of converting the sugar into a highly polar compound, such as a carboxylic acid.

Target of optimization and external constraints. In a previous work on the design of the pentose phosphate cycle [4], we stated that the target of its design optimization was the maximization of flux and the minimization of transition time. If the same principle can be applied here, then the flux to be maximized is the flux of ATP production and the minimization of the response time to achieve its steady state. However, regarding glycolysis (a pathway of carbon degradation whose liberated energy must be coupled to make ATP), one can consider two different targets for its optimization, (a) the maximization of the flux of ATP production, as well as its metabolic response time to reach the steady state (kinetic target), and (b) the energetic yield of ATP production (thermodynamic target).

Each of these variables is a consequence of one of the following different external constraints: (a) a constant chemical affinity (constant concentration of substrates and products); (b) a constant input flux. The maximization of flux can only be achieved at constant affinity, since under conditions of a constant input imposed externally, the flux is not a variable which the system can optimize internally, and it can only change its energy yield (ATP produced/glucose). In contrast, under a constant substrate concentration (total amount of substrate infinite), it has no meaning to maximize the ATP yield from glucose. Regarding the different cells and organisms in nature, examples of these two kinds are evident, which are evidence that these two targets have played a role in evolution. In the following section, reasoning has been developed on the basis of the maximization of ATP flux production as the optimization target; thereafter, the other possibility is dealt with.

ATP traffic. Although a net ATP production is the target of glycolysis, the spending of some of it to transfer phosphate groups can also be necessary as a requisite of some chemical constraint; then, such an ATP expense could give an additional exergonism which is, in principle, good for kinetic purposes. The questions are as follows. How many ATP molecules must be spent? How many ATP molecules can (or must) be produced, and where?

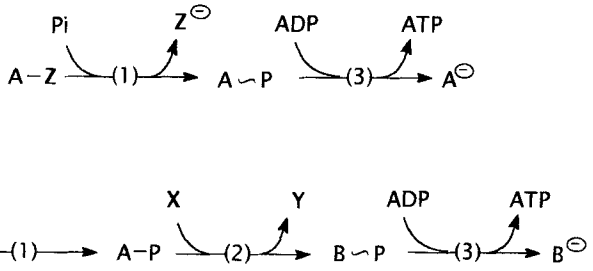
Sugar phosphorylation. There are two reasons for spending ATP in the first reaction of the pathway, to supply a great exergonism at the beginning, and to provide the polarity needed to the sugar. These two purposes could be achieved, in principle, by other means, for example, if glucose is the substrate; then the aldehyde group in C1 can be oxidized to a carboxylate to give gluconate (GlcA) without any phosphate traffic; this mechanism actually occurs at the beginning of the pentose cycle on glucose 6-*P*, and directly on glucose in some Archaebacteria (see below a discussion on that pathway). This reaction is exergonic with an equilibrium constant (q_i) = 100, and it would also solve the problem of polarity, since GlcA is a highly polar compound. Nevertheless, this kind of design is very inconvenient, as it eliminates a means of producing net ATP (Fig. 3). The polarity should, therefore, be supplied by a phosphorylation at the first step of the pathway; it can be done by means of one of the following four mechanisms (see Table 1): (a) Phosphorylation

Table 1. Different possibilities to phosphorylate the starting sugar. Values of ΔG^c were calculated as described in Thermodynamic design. ΔG^c of ATP hydrolysis is −49.22 kJ/mol. Italic names denote hypothetic enzymes.

Reaction	Enzyme	Global ΔG^c
		kJ/mol
(a) Glc + ATP → Glc6*P* + ADP	hexokinase	−20.9
(b) Glc + ATP → Glc1*P* + ADP Glc1*P* → *Glc6P*	glucose 1-kinase phosphoglucomutase	−20.9
(c) Glc + P_i → Glc1*P* Glc1*P* → Glc6*P*	glucose phosphorylase phosphoglucomutase	+28.32
glycogen + P_i → Glc1*P* Glc1*P* Glc6*P*	glycogen phosphorylase phosphoglucomutase	−4.15
(d) Glc + P_i + NAD^+ → GlcA1*P* + NADH + H^+ GlcA1*P* + ADP → GlcA + ATP	glucose dehydrogenase (NAD^+) gluconate 1-kinase	+2.52

on any OH group of the molecule, other than C1, with ATP expenditure. (b) Phosphorylation on C1, with ATP expenditure. (c) Phosphorylation on C1 with inorganic phosphate, without ATP expenditure, by means of hypothetic glucose phosphorylase equivalent to glycogen phosphorylase. (d) Phosphorylation on C-1 by inorganic phosphate coupled with aldehyde oxidation, to give GlcA1*P*, which could eventually be coupled with a kinase to produce ATP, as occurs in the reaction of glyceraldehyde 3-*P* (Gra3*P*) dehydrogenase. Mechanisms (b) and (c), which involve a phosphorylation on C1, must be followed by a mutase reaction to change the phosphate to another position, since the C1 must be available to produce ATP through the acyl-phosphate mechanism. Option (b) makes, therefore, no sense, and must be discarded, since the same result can be obtained through option (a) in just one step. Option (c) is allowable on glycogen (ΔG^c = −4.5 kJ/mol), but it is hardly possible on glucose (ΔG^c = +28.32 kJ/mol), since it does not account for the exergonism necessary at the beginning of the route. Option (d) is good for

Scheme 1. Metabolism of a sugar to produce ATP.



the purpose of ATP production, but it is not kinetically good placed at the beginning of the pathway since it is a little endergonic (ΔG^c = +2.52 kJ/mol). The best mechanism from a kinetic point of view, taking into account the conclusions of the first part [14] (Fig. 1), is option (a). This mechanism preserves the C1 to be used later for net ATP synthesis, and does not imply a net ATP expenditure because it can be recovered later by means of the phospho*enol* mechanism.

Phosphorylation (ATP synthesis) at the substrate level. A reaction capable of making ATP must have a high equilibrium constant in order to be able to work under a wide range of concentrations of substrates and products, and so to be useful to the cell under a wide range of conditions. Regarding chemical reaction mechanisms, the transfer of a phosphate group to ADP to make ATP can only be possible from a compound with a good leaving group, i.e. a group R attached to the molecule capable of yielding a stable negatively charged product R^-; (for example, a phosphate cannot be transferred to ADP from an ester phosphate -CH_2O-(P) because the -CH_2O^- is a poor leaving group].

An obvious condition for a net ATP synthesis is that the phosphate group is previously transferred to the intermediate as inorganic phosphate; the possible mechanisms for this transformation are represented in Scheme 1. The phosphate must be attached to an intermediate of the pathway; this can be done by means of substitution or addition. In the first case, the whole process occurs in two steps, but the initial substrate must have

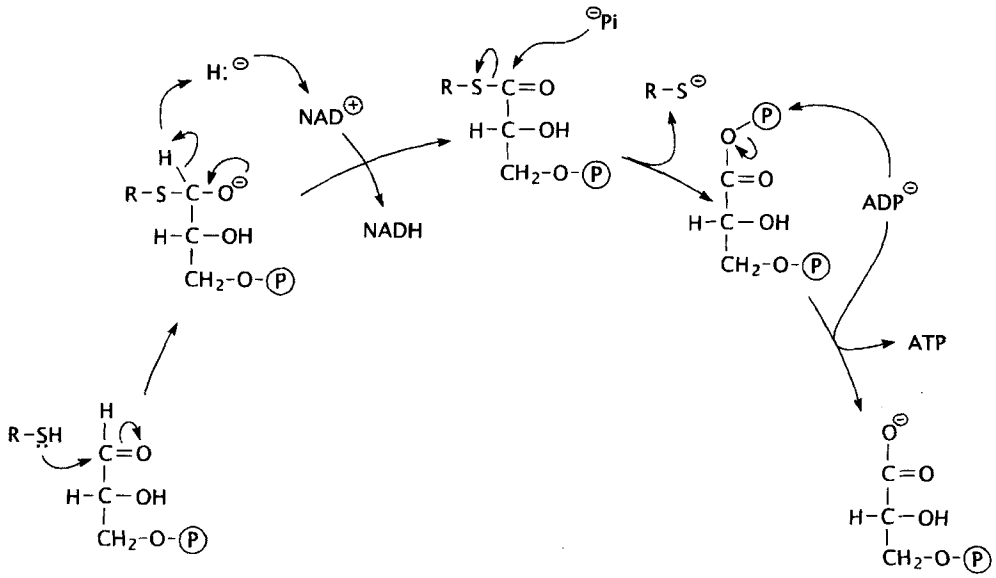


Fig. 3. Phosphorylation at the substrate level by the acylphosphate mechanism. This procedure is based on the addition of a phosphate (preceded by the addition of a sulfhydryl group) to the double bond C=O of the carbonyl group of an aldehyde; a further oxidation, which is chemically allowable with NAD^+, converts it into a carboxylate which can then act as a leaving group for the transfer of phosphate to ADP. The R-SH reagent supplied by a cysteine residue in the enzyme gives the $R\text{-}S^-$, a good leaving group which allows the incorporation of P_i.

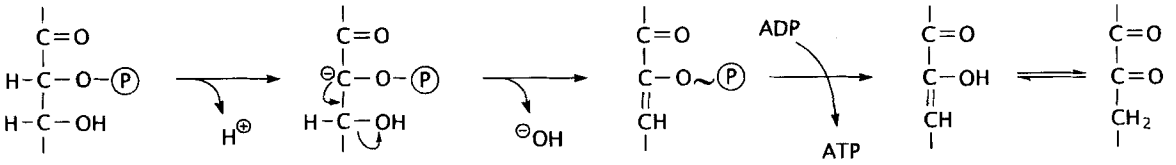


Fig. 4. Phosphorylation at the substrate level by the phosphoenol mechanism. This mechanism involves two steps: (a) formation of the phosphoenol group by enolase, and (b) transfer of the phosphate to ADP by a kinase. The loss of water in the first reaction, promoted by the loss of a proton, requires a carbonyl to be adjacent in order to stabilize the carbanion. The *enol* group is a good leaving group for the phosphate transfer because of its resonance with the double bond C=C.

a leaving group *Z*. In the second case, there is no leaving group in the initial substrate, and the whole procedure involves three steps; attaching of the phosphate group, conversion of the resulting phosphorylated intermediate into a different one to make a leaving group, and the transfer of phosphate to ADP. Now we shall prove that, in the metabolism of a sugar (monosaccharide), ATP can be made only through the acylphosphate mechanism, or whatever else is equivalent (Fig. 3). This property is stated by the following theorem:

Theorem 1. Under the usual thermodynamic conditions of life (with an equilibrium constant reasonably high), the acylphosphate mechanism (Fig. 3), or any mechanism derived from it, is the only one capable of achieving a net ATP production at the substrate level, from a sugar (monosaccharide) metabolism.

Proof. Any mechanism for a net ATP production is represented in Scheme 1, and must satisfy the following conditions.

Condition A. Reaction 1 (phosphate incorporation) can be one of the following: (a) substitution; (b_1) addition to double bond C=C; (b_2) addition to a double bond C=O.

(a) If this is a substitution, then *A* has to be attached to a leaving group (Z). The carbonyl of the sugar could promote a leaving group, for phosphate incorporation, as R-SH does (Fig. 3), but it leads to the same acylphosphate reaction.

(b_1). An addition to a double bond C=C could be, in principle, a possible mechanism. The problem is that the production of such a double bond in a sugar leads to an *enol* intermediate which is highly unstable; such an intermediate could be, however, stabilized by one enzyme, but it could not avoid the global reaction to be highly endergonic, and so very inconvenient. For example, the hypothetic phosphorylation through a C=C bond of glycerate (Gri) by inorganic phosphate (Gri + P_i → Gri3*P*) is chemically bad, having a free energy change highly positive (ΔG^c = +31.32 kJ/mol), which is not so different from a direct synthesis of ATP as ADP + P_i → ATP + H_2O (ΔG^c = +49.22 kJ/mol), without any coupled transformation of a carbon compound. Therefore, a mechanism to synthesize ATP based on an addition of P_i to a double-bond C=C in a sugar must be discarded as unlikely.

(b_2). An addition to a double bond C=O is a mechanism truly possible in sugars, and indeed the only possible, as they have this carbonyl group.

Condition B. It must be a net chemical transformation of A into B, as Scheme 1 shows because, if not, then only the reaction ADP + P_i → ATP would occur, where A would only serve as a catalyst, and has no thermodynamic meaning. Therefore, conversion of ADP + P_i into ATP would require an external energy source, here represented by X → Y, such as the conversion of NAD^+ into NADH.

Condition C. The intermediate which will transfer the phosphate to ADP must have a good leaving group (A^- or B^- must be stable). The possible high-energy groups that could play this role are shown in Table 2. Only acylphosphate (Fig. 3) and *enol*phosphate (Fig. 4), as carbon, can suffice; other groups such as thioesters, or guanidine phosphates could also be used, but only as enzyme active-site derivatives. The difference between them is that the acylphosphate can account for net synthesis of ATP, since it also guarantees phosphate incorporation (by addition to the C=O group), but the enolphosphate mechanism does not. Then, since the only possible mechanism to produce a net ATP involves a carboxyl group, and there is none in a sugar, it has to be made by oxidation of another group, which proves the theorem. Other mechanisms which operate in cell metabolism to make ATP, such as the *P*-pyruvate (Fig. 4), do not account for a net synthesis of ATP, as they cannot justify the previous bond of the phosphate group.

Table 2. Group-transfer high potential compounds.

Group	Transfer of	Leaving group	Examples
Pyrophosphate	phosphate	phosphate	hexokinase
Thioester	acyl	thiol	amino-acid *N*-acetyltransferase
Thioester	thiol	carboxylate	propionate CoA-transferase
Acylphosphate	acyl	phosphate	acyl-CoA synthetase
Acylphosphate	phosphate	carboxyl	phosphoglycerate kinase
*Enol*phosphate	phosphate	enol	pyruvate kinase
*Enol*phosphate	enol	phosphate	3-phosphoshikimate 1-carboxyvinyl-transferase
Guanidinophosphate	phosphate	guanidino	creatine kinase

Redox cycles. According the hypothesis of anaerobiosis, the oxidation of aldehyde to carboxyl to make ATP must be balanced with one reduction reaction, composing a redox cycle. We shall now examine what redox cycles can occur on every sugar (see Fig. 5 and Table 3).

C_2 sugar. The sugar of two carbons (glycolaldehyde) does not occur in metabolism as a free intermediate, most probably because it is very reactive, and so it could produce many uncontrolled reactions. In any case (Fig. 5), a redox cycle is not possible on a C_2 sugar.

C_3 sugar. The redox cycle leads us to the familiar sequence from Gra3*P* to lactate, with net production of one ATP, as occurs in standard glycolysis (see Fig. 2).

C_4 sugar. A C_4 sugar could be metabolized in two ways; by breaking down into two C_2 sugars, or without breaking. In the first case one redox cycle, with ATP production, is possible by means of combination between the two parts (Fig. 5), but the *enol*phosphate mechanism cannot occur and, thus, the phosphate spent previously cannot be recovered; the latter case gives the

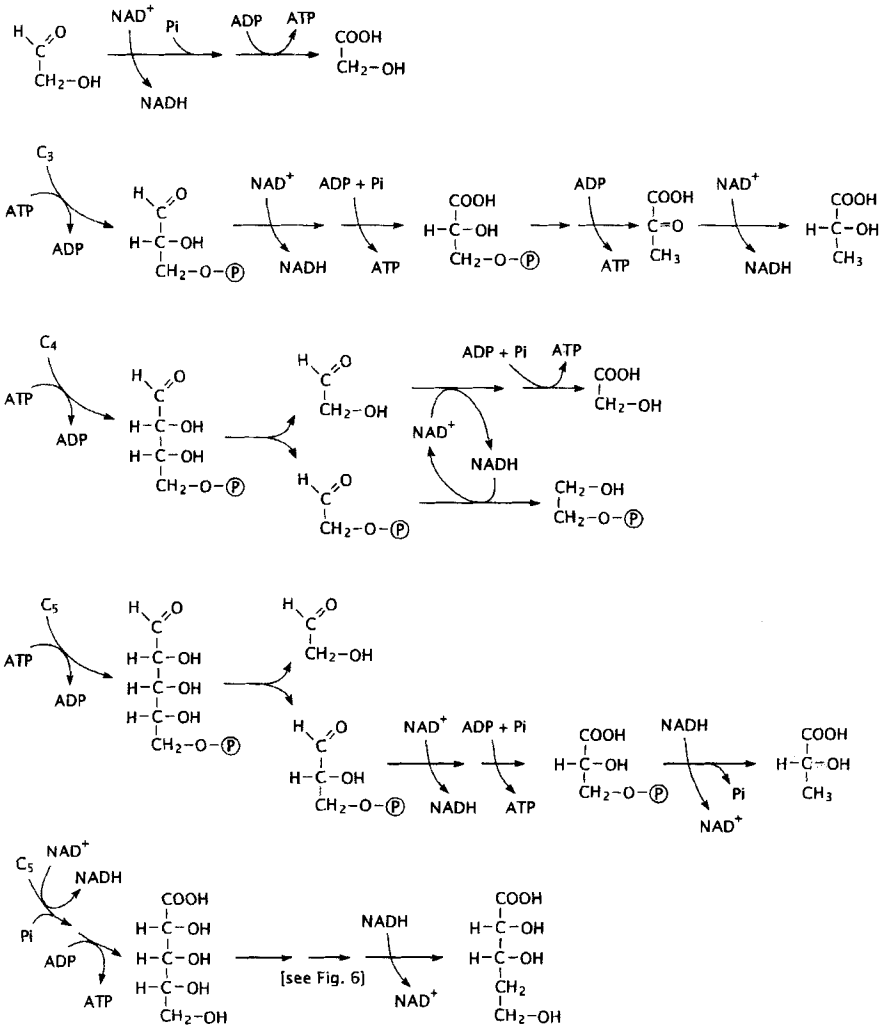


Fig. 5. Metabolic possibilities for sugars with a different number of carbons to support redox cycles and to produce ATP. The result of this analysis is summarized in Table 3. It is assumed that the C_2 sugar (glycolaldehyde), as a highly polar compound, does not need to be phosphorylated. A C_2 sugar cannot support a redox cycle because the NADH reduction of $-CH_2-OH$ to $-CH_3$ is not possible since HO^- a poor leaving group. A C_3 sugar yields the familiar sequence from Gra to lactate shown in Fig. 1. A C_4 sugar has the same possibilities as a C_3 sugar. A C_5 sugar, metabolized by breaking down into C_2 + C_3, has the same possibilities as a C_3 sugar since the C_2 part cannot support a redox cycle, nor can the C_3 part have a second reduction to balance the oxidation of the C_2 part; a C_5 sugar metabolized without breaking down cannot yield more than one ATP (by means of the acylphosphate mechanism) according to Theorem 1 (see Fig. 3). The different possibilities for a C_6 sugar (Table 3) are easily derived from the cases shown here. The expense of ATP at the beginning of each pathway is the least possible, in order that the resulting products meet the hypothesis of polarity.

same result as a C_3 sugar. A C_4 sugar has, therefore, no more metabolic possibilities than a C_3.

C_5 sugar. Metabolism of a C_5 sugar can give just one ATP; this property is described by the following theorem:

Theorem 2. Under the same conditions as Theorem 1, it is not possible to produce more than one net ATP in the metabolism of any sugar with fewer than six carbons, under the anaerobiosis hypothesis.

Proof. A sugar with five carbons could, in principle, be metabolized by breaking down into C_2 + C_3, or without breaking. In the first case, it can only yield one ATP (on the C_3 part) because the C_2 itself cannot support a redox cycle, nor can the C_3 part be reduced twice to complete the cycle of the C_2 part (Fig. 5). Through a mechanism without breaking down, one ATP can be produced by the acylphosphate mechanism with oxidation of C1 to a carboxyl; this oxidation must be balanced with reduction on another carbon. The production of a second ATP (if possible), also through an acylphosphate, would have to occur by oxidation of the other end of the sugar; then, a previous oxidation must be carried out to convert the hydroxymethyl into aldehyde, and a second oxidation must occur afterwards to produce the carboxyl via the phosphorylation mechanism. These two oxidations need two reduction reactions to balance the NAD^+/NADH ratio, according to the anaerobiosis condition. Thus, the whole process would imply three redox cycles, which means three reductions by NADH. Let us see now what features a molecule must have to be able to support NADH reduction. This is not possible directly on a -CH(OH)- group to convert it into $-CH_2-$, since HO^- is a poor leaving group [-CH(OH)- is not chemically reduced by $LiAlH_4$, a powerful $H:^-$ donor]. Fig. 6A shows the general mechanism for such a reduction. The reduction by hydride depends, thus, on the previous formation of a keto group. Three carbons are necessarily involved in the conversion $-CH(OH)- \rightarrow -CH_2-$, which accounts for a reduction by one net NADH. However, each set of three carbons does not need to be independent allowing overlapping among them (Fig. 6B); thus, three consecutive carbons account for one reduction, four carbons make possible two reductions, and five

Table 3. ATP yield and redox cycles in the metabolism of sugars, according to their number of carbons. The first reaction must, in either case, supply a high degree of polarity as e.g. oxidation to make a carboxyl, or ATP spent for phosphorylation. ATP/C means net ATP produced/carbon of the starting substrate sugar. The standard glycolysis shown in Fig. 2 (C_6 metabolized as $C_3 + C_3$), has the highest ratio ATP/C, and the lowest number of steps/ATP produced, which means the highest exploitation of material using the fewest number of different enzymes.

Starting substrate	Metabolized as	ATP				Redox cycles	Steps	Steps/ATP	ATP/C
		spent	produced						
			enol-*P*	acyl-*P*	net				
C_2	C_2	0	0	1	1	0	–	–	–
C_3	C_3	1	1	1	1	1	7	7	0.33
C_4	C_4	1	1	1	1	1	8[a]	8	0.25
	$C_2 + C_2$	1	0	1	0	1	5	–	–
C_5	C_5	0	0	1	1	1	4	4	0.2
	C_5	1	1	1	1	1	9[a]	9	0.2
	$C_2 + C_3$	1	1	1	1	1	8	8	0.2
C_6	C_6	0	1	1	1	1	4	4	0.16
	C_6	1	1	1	1	1	10[a]	10	0.16
	$C_2 + C_4$	1	1	2	2	3	14[a]	7	0.33
	$C_3 + C_3$	2	2	2	2	2	11	5.5	0.33
	$C_2 + C_2 + C_2$	1	0	1	0	1	6	–	–

[a] Mechanisms which could have had fewer steps, discounting some mutase reactions.

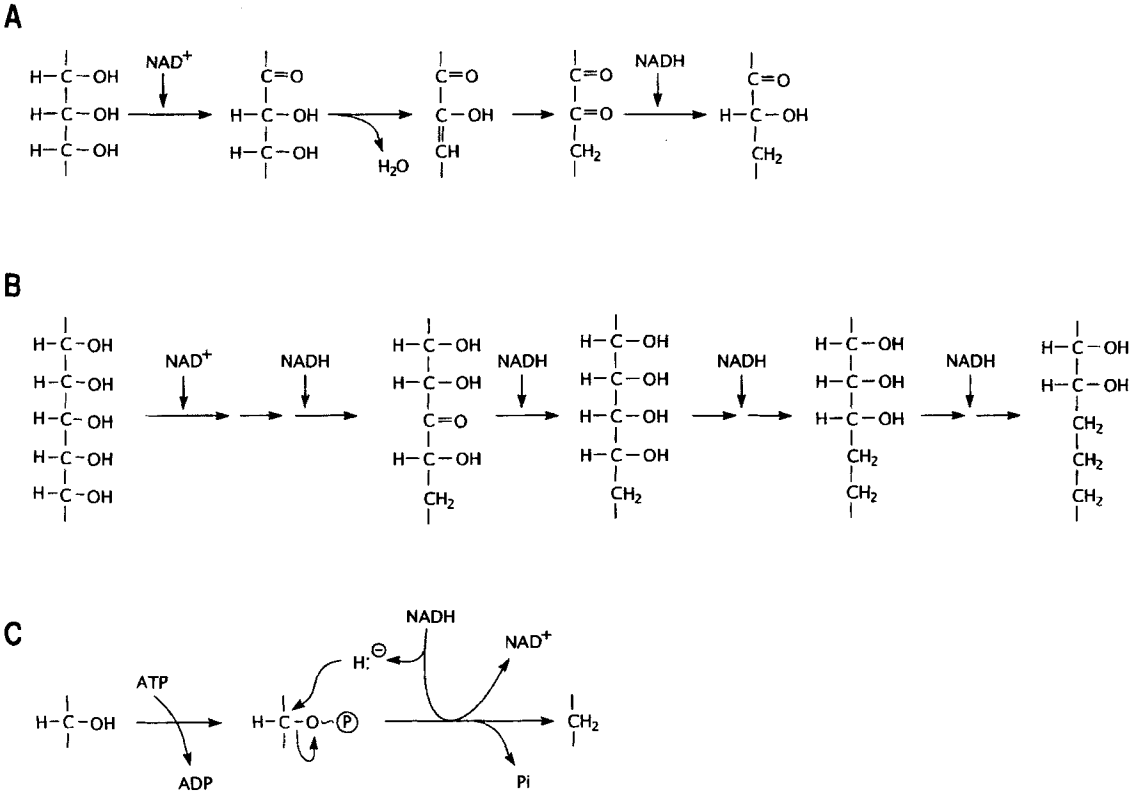


Fig. 6. Reduction of -CH(OH)- to -CH_2- in a sugar by NADH. Illustration of the proof of Theorem 2. (A) The minimal general mechanism for such a reduction involves three carbons of the sugar. The group -CH(OH)- cannot be reduced directly to -CH_2- by NADH since hydroxyl is a poor leaving group. Thus, the reduction by hydride must be preceded by a enolic reaction, which depends on the previous formation of an adjacent keto group. The first carbon of the set can be C1 of the sugar, where there is already a carbonyl; this simplifies the process, requiring less redox cycles. (B) Scheme showing the possible overlapings allowing several reduction reactions on the same set of carbons; three consecutive carbons can account for one reduction, four carbons for two reductions and five carbons for three reductions. (C) The introduction of a phosphate, as a good leaving group, by spending ATP, can allow the reduction by NADH directly.

make possible three. There, the first carbon of the set can be C1 of the sugar, where there is already a carbonyl, but the last carbon cannot be one of these series, since it is converted into a carboxyl by the acylphosphate mechanism, and the last one of the set becomes a methylene. Therefore, any sugar must have at least six carbons to support this chain of reactions, which proves the theorem. This reasoning has been developed on the hypothesis of no extra ATP spending; the complicated reductive process (Fig. 6A, B) could be largely simplified by spending ATP (Fig. 6C) to introduce a phosphate, which is a good leaving group and allows the direct reduction of -CH(OH)- by NADH, but it would mean two ATPs spent to gain one, and it is not a solution. In fact, many of the chemical mechanisms considered in this work as impossible or unlikely, could be possible by a procedure involving ATP spending.

C_6 sugar. There are four possibilities for metabolizing a C_6 sugar; (a) without breakdown, (b) breaking down into $C_3 + C_3$, (c) breaking down into $C_2 + C_4$ and (d) breaking down into $C_2 + C_4$, followed by a further breakdown of the C_4 into two C_2. The analysis of every possibility leads us to the following conclusions.

(a) Metabolism of C_6 without breakdown produces two ATPs by means of the acylphosphate mechanism; the whole pathway (not shown) has 15 steps, including five redox cycles, as minimum, for a net gain of two ATPs, and its kinetic yield is very poor.

(b) Breaking down of C_6 into two C_3, as the mechanism in the standard glycolysis scheme (Fig. 2), is clearly the simplest mechanism, since it is the only way where any sugar yields two ATPs with only two redox cycles. The symmetry of this solution also means that these two redox cycles are carried out by the same set of enzymes; only one enzyme (Gra3*P* dehydrogenase) works with NAD^+, and also only lactate dehydrogenase works with NADH to close the redox cycle, there being no competition among them, which also enhances the kinetic efficiency of the system, as we have previously shown [4].

(c) The breaking down of C_6 into $C_2 + C_4$ can also yield two ATPs since the C_4 part could support an extra reduction, allowing the oxidation of the acylphosphate mechanism in the C_2 part. However, this is not a good solution, since the complete procedure (not shown) has a number of bad features as follows: 14 steps (longer than the $C_3 + C_3$ solution, to get the same result), and three different redox cycles; this means three different enzymes in the $C_2 + C_4$ solution competing for the same pool of NAD^+ and also three competing for the NADH pool (instead of only one of each in the $C_3 + C_3$ solution), which produces a low kinetic efficiency [4].

(d) The breaking down of C_4 to give two C_2 makes no sense, since, although a redox cycle (with the consequent ATP production) is possible by combining the metabolism of two C_2 parts (Fig. 5), the same result is obtained with the direct (and thus, simpler) metabolism of C_4; therefore, this path can also produce only one ATP. This analysis of all possible cases for every sugar is summarized in Table 3. C_3 metabolism, and so the breakdown of C_6 into two C_3, produces the maximum possible ATP with the least possible number of carbons and different enzyme reactions. This is also a way to simplify the design of the pathway, since it allows the production of two ATPs under just two (stoichiometric) redox cycles, but only one from the kinetic point of view. Since one triose molecule can only come from the breakdown of a larger sugar, it is much better from a C_6 sugar than a C_5 sugar, since C_6 sugars yield two trioses, which maximizes the exploitation of the material.

Symmetry and stoichiometric simplicity. Symmetry in the structure of a metabolic pathway is defined as a particular kind of design where two parts of the pathway are driven through the same sequence of reactions, which are then joined or, conversely, a given compond is broken in two parts which then are converted by the same chain of reactions. This is a frequent design in metabolism, as has been previously shown in the pentose phosphate cycle and Calvin's cycle in photosynthesis [1–4]; a symmetrical design can be seen in other pathways, such as the biosynthesis of cholesterol (see, e.g. [20]).

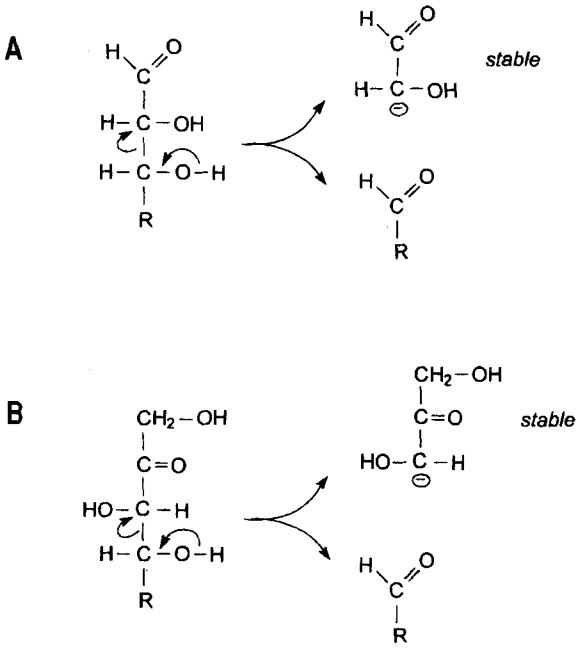


Fig. 7. Breakdown of an aldo-sugar, and of an keto-sugar. Aldolic breakdown of a sugar giving two sugars. (A) Breakdown of an aldo-sugar (e.g. glucose) between C2 and C3; (B) breakdown of a keto-sugar (e.g. fructose) between C3 and C4. The carbonyl group at C1, or C2, is always necessary to stabilize the resulting carbanion; thus, the breakdown can only occur between the carbon adjacent to the carbonyl, and the next one. Aldolase catalysis is based on this mechanism.

The symmetrical design of a pathway has a number of advantages; it provides more kinetic efficiency [4], it saves enzymes, and it takes better advantage of the intermediate pools, which result in a more economic and efficient machinery. The standard glycolytic design (Fig. 2) has the maximum possible symmetry. In effect, for the $C_6 \rightarrow C_3 + C_3$ stoichiometry, glucose has to be isomerized to fructose, since only a *keto*-sugar can be broken between C3 and C4 to give two triose molecules (Fig. 7). These two triose molecules are not the same because one of them must be an aldehyde, and the other a ketone, thus the reaction of triose phosphate isomerase is always necessary to join the two symmetrical halves. The breaking down of the *keto*-hexose bisphosphorylated at C1 and at C6 produces Gra3*P*, then a mutase reaction is necessary to pass the phosphate to C2 for the *enol*phosphate mechanism to operate (see Fig. 4).

This mutase step might be avoided if the product of aldolase were Gra2*P* instead of Gra3*P*; then, the first reaction of glycolysis should have been Glc → Glc5*P*. However, this is not possible, because the OH at C5 is not available in the cyclic structure of glucose (the major structural form). Moreover, Gra2*P* would have been a poor substrate for the Gra*P* dehydrogenase reaction (see Fig. 3), since the proximity of the phosphate at C2 would make the P_i incorporation more difficult due to the repulsion between negative charges at the adjacent position. The cellular reaction avoids this problem since the phosphates are more distant in positions 1 and 3. In any case, C5 phosphorylation would not solve anything, because the other C_3 half, coming

Table 4. Standard free energy change at cellular conditions (ΔG^c) of reactions which make ATP in cellular metabolism at the substrate level. Standard cellular conditions are defined as all substrate and product concentrations (including CO_2) at 0.5 mM, $[H^+]$ = 0.1 μM (buffered), NAD^+ and 25 °C. ΔG^c of ATP hydrolysis at these conditions is −49.22 kJ/mol. Reaction of succinyl-CoA synthetase in the Krebs' cycle makes GTP by means of the acyl-*P* mechanism, as phosphoglycerate kinase.

Enzyme	Reaction	ΔG^c
		kJ/mol
Phosphoglycerate kinase	acyl-*P* + ADP → carboxylate + ATP	−18.90
Pyruvate kinase	enol-*P* + ADP → keto-acid + ATP	−31.70
P-Pyruvate carboxykinase	enol-*P* + CO_2 + GDP → carboxy-keto-acid + GTP	+31.02
Propionyl-CoA carboxylase	carboxy-acyl-CoA + ADP + P_i → acyl-CoA + CO_2 + GDP	+4.31

from dihydroxyacetone-*P* necessarily has its phosphate at C3, so the mutase step is always necessary. C1 and C6 are the only two positions of the hexose available to be phosphorylated. C1 and C2 must be free for the isomerase reaction; therefore C1 can only be phosphorylated after the isomerase step, and C3 and C4 are needed for the aldolase.

Thermodynamic design. A pathway with a good thermodynamic design must have a high global equilibrium constant to allow the pathway to work well with no strong dependence on the mass-action ratio of external substrates and products. This condition is very important in cellular metabolism because antagonistic pathways (e.g. glycolysis/gluconeogenesis) should function independently as a consequence of regulation signals, and not because of changes in substrate concentrations. Every metabolic pathway needs a given high net exergonism (high chemical affinity, $\Delta G \ll 0$) as the necessary condition to work (Eqn 1). This goal is easy to reach if the global equilibrium constant of the pathway is high, but it could not be allowed in some cases because of chemical constraints; then the cell must compensate by adapting their substrate and product concentrations. The thermodynamic design of a metabolic pathway can thus be well described by the profile of the standard free energy values (which is a logarithmic scale of the equilibrium constants) and not the actual values. The information supplied by these actual values is very interesting, but it gives rather different information.

Standard conditions are usually stated at a concentration of 1 M for every reactant. However, for the purpose of this work, i.e. in order that they can be useful as a reference point, a reference state must be used which is as near as possible to the condi-

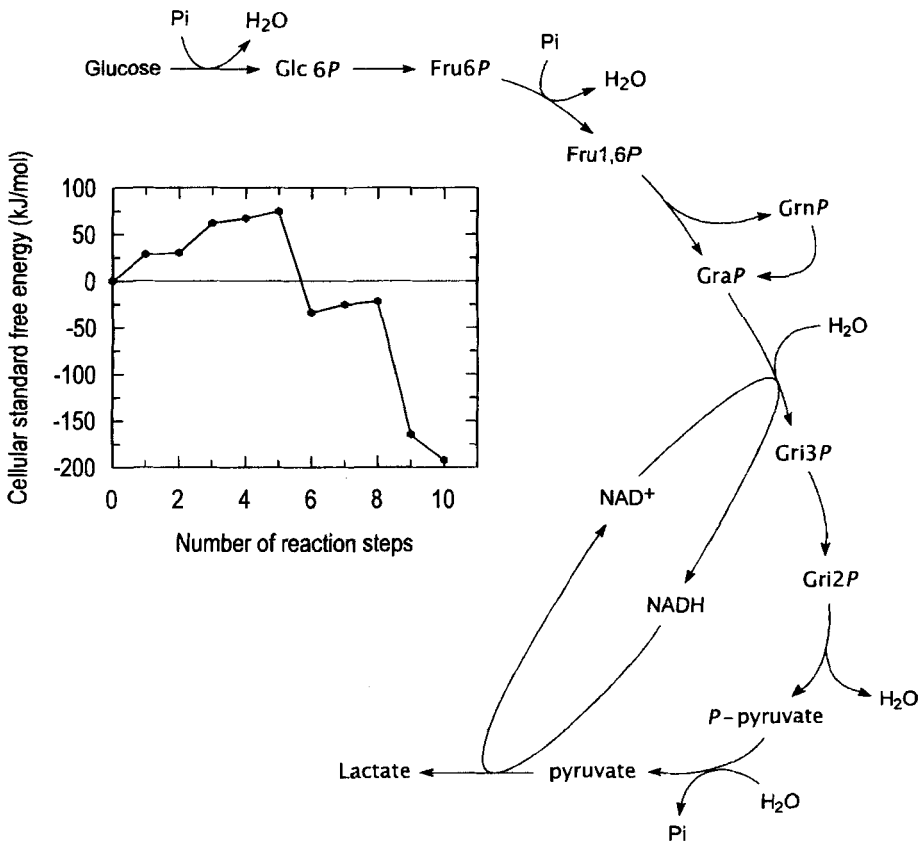


Fig. 8. A glycolytic design without ATP traffic. Hypothetic pathway where glucose is also converted in two lactate, and there is also phosphate traffic, but without involving ATP (synthesis or spending); hexokinase and 6-phosphofructokinase have been substituted by phosphorylases (see Table 1), phosphates entering directly as P_i. There is no phosphate traffic in the reaction of Gra3*P* dehydrogenase and, thus, phosphoglycerate kinase does not exist. Pyruvate kinase has been substituted by a phosphohydrolase; the whole exergonism of this route is obviously much higher than in the regular glycolysis, since there is no net ATP production, but its thermodynamic profile is typical of antiglycolytic design as stated in the former paper [14]. The thermodynamic profile, as ΔG^c along the pathway, is shown in the inset. Note that although this design has a higher whole exergonism than the regular glycolysis (as there is no ATP production), its kinetic efficiency must be worse according to its poor thermodynamic design.

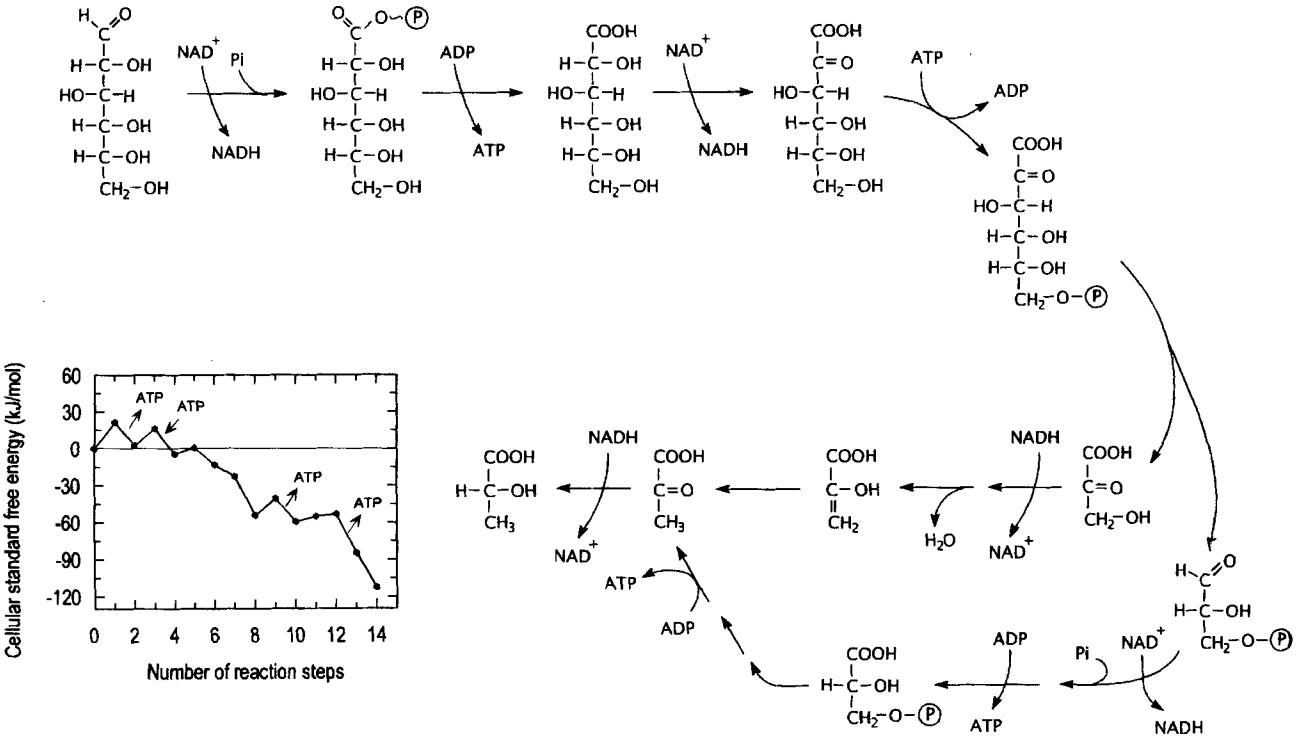


Fig. 9. Antiglycolytic design. A (hypothetical) chemically feasible pathway to convert glucose into lactate. It accounts for all chemical hypotheses stated in this paper, and there is no unjustified reaction according to the purpose of glycolysis. This pathway has the same stoichiometric ATP yield, but the reactions involved in the ATP traffic have been placed in different sites to give poor kinetic efficiency: the exergonic steps (involving ATP expense) are nearest to the end as possible, and the endergonic steps are nearest to the beginning as possible, thus giving a poor thermodynamic design (see the inset). This pathway is the worst glycolytic design which was found, and it is a good proof that the regular glycolysis is the result of an optimization process carried out through metabolic evolution.

tions under which our reactions occur, since a good design of glycolysis may be quite different according to the working conditions. This is not as important for reactions with the same number of substrates and products, but for reactions such as aldolase in glycolysis, with one substrate and two products, the range of concentration chosen has dramatic effects on the thermodynamic value; a reaction such as A → B + C is favoured in the forward direction at low concentrations and the reverse direction favoured at high concentrations (see a discussion on that in [21]). Our calculations on thermodynamics of glycolysis presented here have been made for 0.5 mM of every compound, including CO_2, which is a mean representative concentration of metabolites that are usually in the range 0.1–1.0 mM, and the NAD^+/NADH ratio is stated as 100, as a mean value between several cells [22]. The proton concentration involved in reactions has not been taken into account because it has been considered buffered at pH 7.0. The temperature is 25 °C, which is also a reasonable mean value between mammals (37 °C), and sea water animals (15 °C). These conditions are termed 'standard cellular conditions' and are denoted as ΔG^c.

The free energy change under standard cellular conditions (ΔG^c) of each reaction has been calculated from its standard value ($\Delta G^{\circ\prime}$) with data taken from [20–27]. Data not available or for hypothetical reactions were estimated from similar real reactions or calculated from data of a real route to reach the same end through a different way, since they are state functions. In branched pathways (Figs 9, 12 and 14) the sequence of steps after the branching point was stated by placing the short branch before the long step; this choice is abitrary but it does not influence the results nor the conclusions, as the most important feature of a good thermodynamic design is to have a high exergonism in the first steps, and in those pathways the branching is always at the end.

The thermodynamic profile of the regular cellular glycolysis (Fig. 2) shows many features that coincide with the predictions of the theory. (a) ATP synthesis mechanisms are placed as nearest to the end of the pathway as chemically possible, and (b) an important exergonism has been supplied as near as possible to the beginning of the pathway by means of ATP expense (recovered later). The reason for such expenditure can be well understood: Fig. 8 shows a hypothetical 'glycolytic' pathway with no ATP traffic is shown; it produces a higher global exergonism, obviously but in comparing the effect of removing the ATP traffic on the thermodynamic profile of this route with those of the regular glycolysis (Fig. 2), it lacks the high exergonism at the beginning, and it is closer to the typical antiglycolytic (non optimized) profile.

In standard glycolysis the two reactions coupled to ATP synthesis, phosphoglycerate kinase and pyruvate kinase are highly exergonic with a global $\Delta G^c = -18.9$ kJ/mol and -31.7 kJ/mol, respectively, in good agreement with the hypothesis stated above. The high exergonism of the last reaction, lactate dehydrogenase, with $\Delta G^c = -13.8$ kJ/mol cannot be avoided because of the highly negative reduction potential of NADH. This, however, does not alter any theoretical conclusion, as the really important feature for a good thermodynamic design is to have exergonic steps at the beginning of the pathway, not to avoid them at the end [14]. The exergonism/glucose molecule is, in each case, twice these values, due to the stoichiometric ratio.

In the preceding paper [14] we have defined antiglycolytic design as a design of glycolysis that has a thermodynamic profile inverted with respect to the optimum design (see Fig. 1b). The theoretical analysis of such a design demonstrated that it has very poor ATP flux. The possible design of glycolysis with no ATP traffic discussed above (Fig. 8) with phosphorylases instead of kinases in the first steps has such a thermodynamic

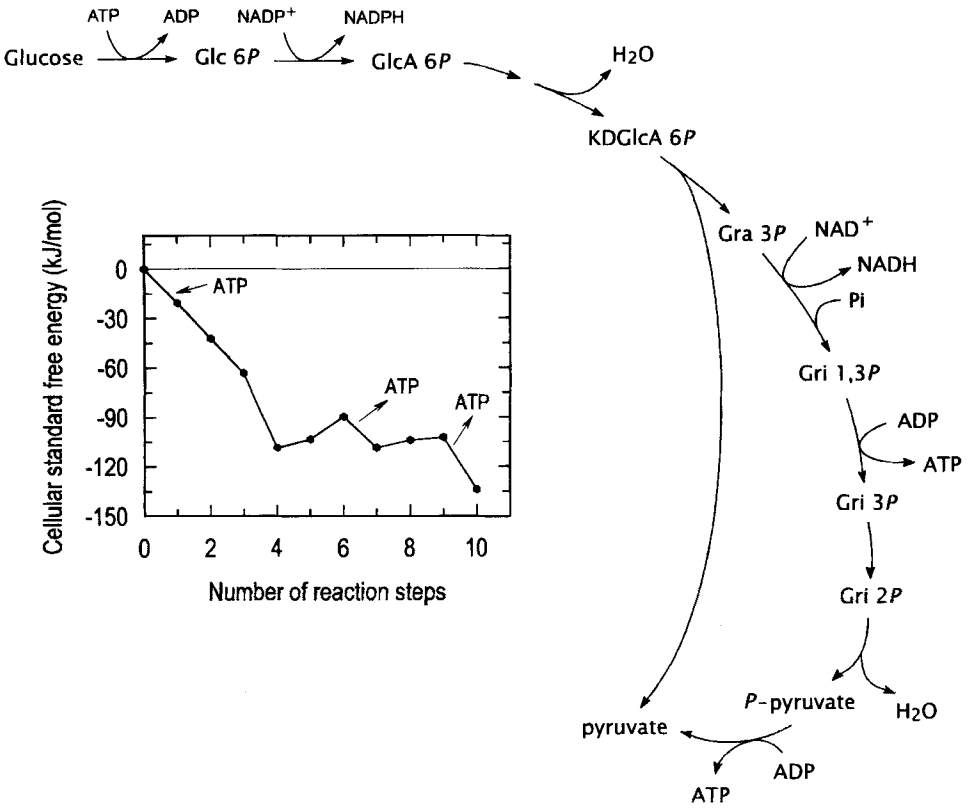


Fig. 10. Entner-Doudorof pathway. This route that occurs in some bacteria [28] is an example of glycolysis paleometabolism; it has a non-symmetrical design and yields only one ATP/glucose. However, its thermodynamic design, shown in the inset, is good, which could explain its preservation as a local basin in the optimization landscape. GlcA, gluconic acid; KDGlcA, 2-keto, 3-deoxygluconic acid.

profile. However, that pathway is not an alternative solution to the problem presented here since it does not make ATP. In Fig. 9 we present a hypothetical pathway which converts glucose into lactate with the same net ATP yield as the regular glycolysis, with a thermodynamic profile very similar to the bad one shown in Fig. 1b. This scheme (the most inappropriate model of glycolysis we have been able to derive), although not found in living cells, is chemically possible and might have occurred during evolution of metabolism. In either case, the real chemical possibilities of these poorly optimized designs, which are different from that of modern cells, are further proof that modern glycolysis is the result of a non-trivial process of metabolic optimization carried out in the course of biological evolution. Note that the main differences among these designs are in the first steps, which is where they are really important as discussed above.

It can, therefore, be concluded that the design of the regular cell glycolysis has solved the problem of ATP synthesis anaerobically, with a high energy yield, the best exploitation of carbon material, and a high kinetic efficiency. In addition, also regarding its metabolic efficiency for other purposes, this design offers great chemical possibilities for intermediates to be coupled with many other metabolic processes, and much thermodynamic freedom for such couplings, without loss of kinetic efficiency.

Other alternative designs of glycolysis in living cells. The metabolism of a sugar to obtain ATP under anaerobic conditions, considered as a chemical problem, can have a number of different solutions. Some of these solutions are only hypothetical (e.g. Fig. 9), but other are real pathways that exist in some particular species. These are analysed in this section.

The Entner-Doudoroff pathway. In some Eubacteria, glucose is metabolized via the Entner-Doudoroff pathway (Fig. 10) [28]. This pathway is a typical case of aerobic glycolytic paleometabolism, it is non symmetrical and yields only one ATP/glucose. However, its thermodynamic design is really good, which could explain its preservation as a local basin in the optimization landscape. Archaebacteria are good candidates for paleometabolism. In *Sulfolobus solfataricus* [29] and *Thermoplasma acidophilum* [30], glucose is metabolized through the non-phosphorylated Entner-Doudoroff pathway (Fig. 11), which is a design still less optimized; it is also non symmetrical, there is no net ATP production, and non-phosphorylated Gra occurs, contrary to the hypothesis of polarity. The thermodynamic profile of this pathway is not bad, although not as good as the preceding case (practically a diagonal from glucose to pyruvate), and it raises the possibility that *Thermoplasma* can synthesize glucose by this route working in the opposite direction, when it is energetically allowable [28].

The phosphoketolase pathway. Similar comments can be made on the pentose phosphoketolase pathway [31], an anaerobic route which occurs in heterolactic fermentative bacteria, through which glucose is converted into lactate and ethanol (Fig. 12). This is another case of paleometabolism where the comments of the former cases also apply; it is also a non-symmetrical pathway with a poor energetic yield but with a reasonable thermodynamic profile, which could be another example of a local basin. In contrast, the presence there of the enzymes of the pentose pathway suggests an evolutionary relationship between the two routes.

The fructose pathway. In the specific pathway of fructose metabolism in the liver [32], fructose is converted into Fru1*P*, which is then cleaved to give dihydroxyacetone-*P* (Grn*P*) plus Gra; this last product can then be metabolized through two alternative routes, as Scheme 2 shows. One of these routes is a non-optimized pathway, because Gra occurs without being phosphor-

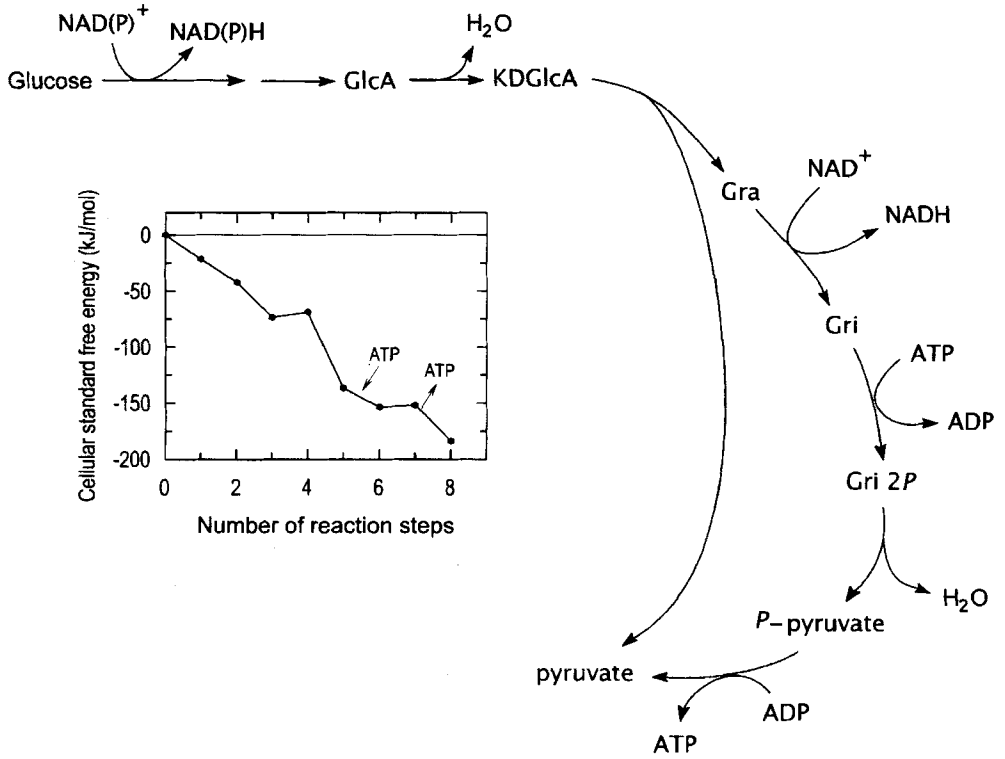
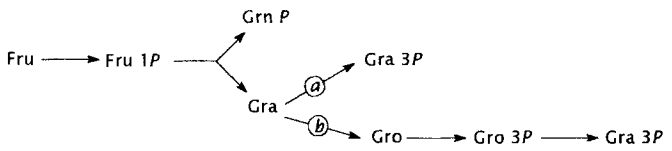


Fig. 11. Non-phosphorylated Entner-Doudorof pathway. A different pathway to convert glucose into pyruvate as occurs in the Archaebacteria *Sulfolobus solfataricus* [29] and *Thermoplasma acidophilum* [36]. This route is an even less optimized pathway than that shown in Fig. 10 and, thus, a more typical case of paleometabolism; it is also non-symmetrical, there is no net ATP yield, and its thermodynamic profile, shown in the inset, is worse than the preceding one.

Scheme 2. Metabolism of fructose.



ylated (violation of the hypothesis of polarity); in option (a) the process is less symmetrical, since there are two different reactions instead of one (triosephosphate isomerase) after the breakdown by aldolase in the standard glycolysis; in addition, this pathway has three new steps to metabolize a new sugar, when only one step (Fru → Fru6*P*) would have been enough. Finally, phosphorylation of Gra, spending an ATP at the fourth step, when it could have been in the second step, makes this design far from optimal from a kinetic point of view, according to previous results [14]. The pathway through glycerol (Gro) in option (b) looks much less optimized.

The phosphotransferase system. Some anaerobic and facultative anaerobic bacteria use an alternative way to phosphorylate glucose and other sugars, using phospho*enol*pyruvate as a phosphate donor instead of ATP, by means of the phosphotransferase system. Also, phosphate transfer is coupled with the transport of sugars (Fig. 13), reviews in [33, 34]. The system has a divergent design in the last steps which makes it possible to phosphorylate a number of sugars and derivatives. This system is an efficient system for transporting sugars [35]. It catalyzes the accumulation of sugars as phosphorylated derivatives, and its energy yield is higher than the standard glycolysis coupled to proton symport (e.g. lactose in *Escherichia coli*). In effect, this pathway makes it possible to produce two ATPs from each glucose instead of 1.66, because 0.33 ATP equivalents must be expended for transport in systems coupled with proton symport.

From the point of view of thermodynamic design, this system is, in principle, good because the coupling between pyruvate kinase and hexokinase is a way to move the high exergonism of the end of the pathway to the first step. However, this poses two important problems for the kinetic yield. (a) The pathway has a loop with *P*-pyruvate intervening in a previous step; it means that the system must be fed in order to work. So, hexokinase is also required to fill the system with intermediates, however, this enzyme must have little activity so that it can easily be replaced by the phosphotransferase system once the pathway is filled, which implies an additional problem of regulation. These steps have to be slow during feeding, and thus its kinetic efficiency can be good when it is running but its metabolic response time is large; this means a very low capacity for a fast response. (b) The phosphate transfer from *P*-pyruvate to glucose is made through five steps. This prolongation of the pathway is kinetically bad, as demonstrated [4, 11]. This kind of design is highly specialized; the metabolic use of *P*-pyruvate is forced, since at least 50% of the *P*-pyruvate (and every preceding intermediate) must be converted into pyruvate, prohibiting their escape to other pathways. This reduction of metabolic possibilities is most probably the reason why the phosphotrasferase system only occurs in anaerobic (or facultative anaerobic) bacteria where glycolytic intermediates cannot be deviated, as the completion of the pathway is necessary to guarantee the redox balance.

The anaerobic pathway of Ascaris. The anaerobic Nematode worm *Ascaris lumbricoides*, a parasite of the human small intestine, has a rather different glycolytic design (Fig. 14) [36]. It includes the gluconeogenic enzyme *P*-pyruvate carboxykinase working at the opposite direction, the coupling with the second part of the Krebs cycle also working in the opposite direction to

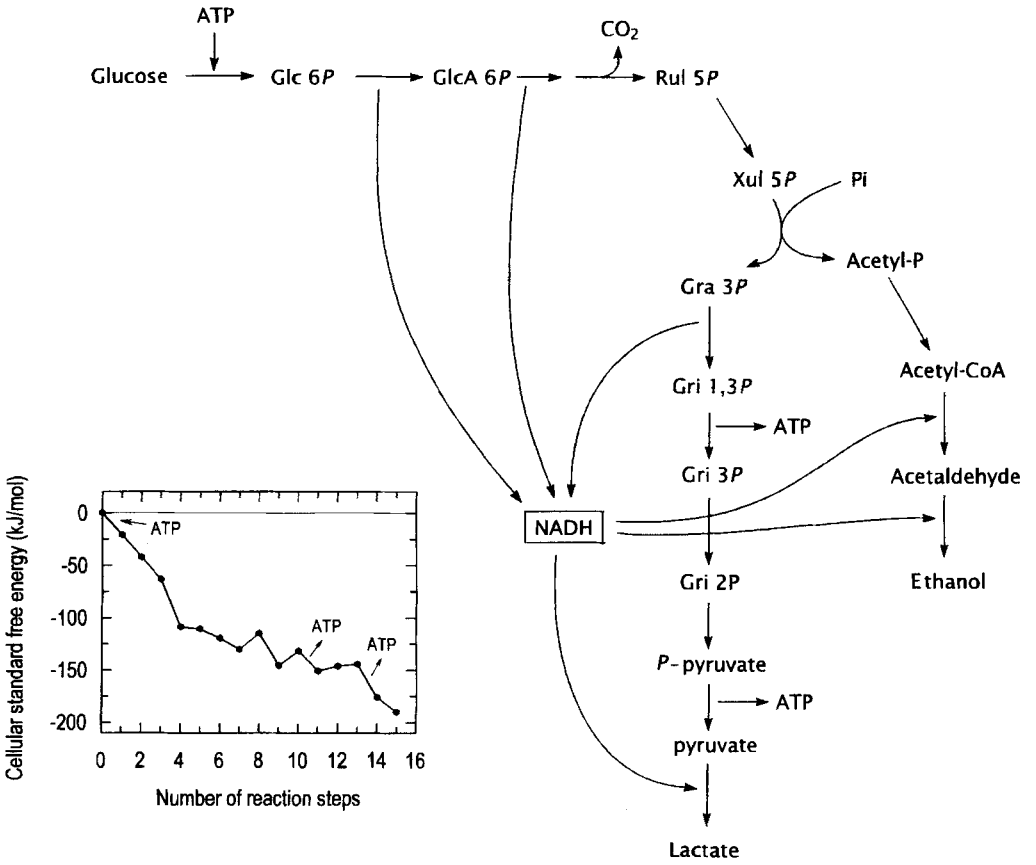


Fig. 12. Pentose phosphoketolase pathway. This route, found in heterolactic fermentative bacteria [31] accounts for the conversion of glucose into lactate, ethanol and CO_2. It can also be considered as a clear case of paleometabolism, joining the pentose cycle and glycolysis. It is also non-symmetrical, produces less ATP, and its thermodynamic design is not optimized, as shown in the inset.

account for the regeneration of NAD^+ (with fumarate reductase instead of succinate dehydrogenase) and the coupling with methylmalonyl-CoA mutase, propionyl-CoA carboxylase and succinyl-CoA transferase, all working in the opposite direction to the regular metabolism in other species. In addition, it has a very complicated stoichiometry as a fraction of flux from *P*-pyruvate must be deviated via acetyl-CoA to account for the anaerobic balance of NAD^+/NADH. The special feature of this pathway is its thermodynamic yield, as it produces 3.33 ATP instead of two from each glucose molecule. The global net stoichiometric equation is

$$3 \text{ glucose} + 10 \text{ ADP} + 10 \text{ P}_i \rightarrow 2 \text{ acetate} + 2 \text{ CO}_2 + 4 \text{ propionate} + 10 \text{ ATP}.$$

Fields [36] has discussed this design, raising the question of why more animals have not made use of this energetically more efficient pathway, and the fact that it is not clear if the pathway necessarily results in an energetic advantage to the organism. The results presented in this paper and previous results [4, 11, 14] allow us to answer those questions.

This pathway is much longer than the regular design (Fig. 2). It has 26 steps, (instead of 11 from glucose to lactate), including the passage of metabolites through the mitochondrial membrane and the conversion of GTP into ATP. We have previously shown [4] that such a long route poses very important kinetic problems; under the same global thermodynamic constraints and the same K_m and V_{max} for all the enzymes, a long chain of reactions has less kinetic efficiency (it supplies less flux) than a short reaction, and this effect is dramatically increased with the number of steps. However, as mentioned above, this effect only applies in cases of a constant chemical affinity of the reaction. At constant input flux, the flux of the metabolic system is externally imposed, and so it does not depend on the complexity of the pathway. Each real biological system operates under one of these two constraints, according to their life pattern, and thus the target for metabolic optimization must be different for each case; the flux can be optimized only in cases of constant affinity, but if the input flux is externally imposed, then it cannot obviously be an optimizable variable. The metabolic pattern of *Ascaris* is a case of constant input as its supply of glucose is externally limited.

Under these conditions, the length of the pathway has another important effect on the kinetic properties that applies under any kind of constraint; the metabolic response time, the time the system requires to fill the pathway with intermediary metabolite steady-state concentrations (or to change those pools in the transient between different steady states). The shorter the chain of reactions of the pathway, the shorter metabolic response time required to reach the same steady-state flux. Thus, a short pathway can account for a quick response to regulation changes, while a long pathway has a slow response. In addition, it must be noticed that the *Ascaris* pathway also has a loop at the end for the succinate/propionate conversion which, as in the phosphotransferase system pathway discussed above, produces important problems of feeding the pathway for starting. This adds more problems to the difficult kinetic design because it needs different (anaplerotic) enzymes to fill the pathway with intermediates, making a transient much more difficult. *Ascaris* can thus obtain more ATP molecules from each glucose molecule at a flux externally imposed and with few possibilities of changing its metabolic rate.

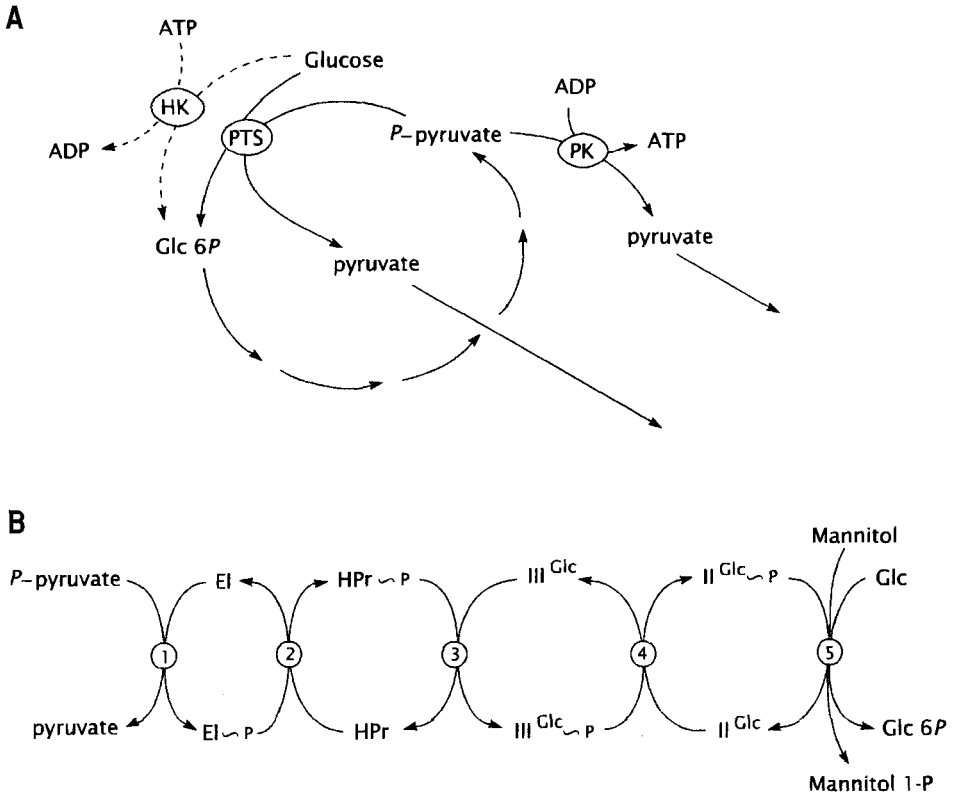


Fig. 13. *P*-Pyruvate phosphotransferase system. This mechanism exists in some bacteria to use the *P*-pyruvate energy to produce Glc6*P* and other sugar phosphoderivatives [33]. (A) Scheme summarized of the reaction integrated with glycolysis. (B) Detail of the system showing the sequence of phosphate transfer from *P*-pyruvate to different sugars. This design could look apparently good, as the high exergonism of pyruvate kinase has been displaced to the beginning of the glycolytic pathway; however, it is kinetically poor because it needs to be fed with glycolytic intermediates to start.

PP_i-fructose 6-phosphate 1-phosphotransferase. This enzyme, found in some higher plants, catalyzes the conversion of Fru6*P* into Fru1,6*P*, using PP_i instead of ATP as the phosphate donor. It has been suggested that this enzyme could play a role in glycolysis as an alternative enzyme to phosphofructokinase [38]. There have been several claims arguing that there is not enough experimental evidence in support of this, and pointing out that the metabolic role of the enzyme is unclear [38, 39]. The free energy change of this reaction at standard cellular conditions is $\Delta G^c = -4.34$ kJ/mol, i.e. near to equilibrium, which means a thermodynamic design less effective than in the standard glycolysis, since it eliminates a high exergonism at the beginning of the pathway. Concerning the energetic yield, this might mean 1 less ATP spent, provided that an independent source of PP_i can be guaranteed, which is not very clear. That reaction could, however, promote another problem related with the global thermodynamics of some pathways; PP_i is a product of several reactions, such as amylopectin, DNA, RNA, and protein synthesis, and fatty-acid metabolism. It is usually hydrolyzed by the ubiquitous enzyme inorganic pyrophosphatase, increasing the exergonism of the previous reaction. Thus, if PP_i was a substrate for other reactions, it would be necessary to maintain a given pool of PP_i, which means having pyrophosphatase activity controlled, then to eliminate the supply of exergonism. This enzyme could therefore modify the thermodynamic features of several pathways; its metabolic role must be carefully studied.

DISCUSSION

The two theorems of glycolytic design presented here are the center of our chemical analysis. Theorem 1 postulates that the acylphosphate mechanism is the only way to produce net ATP at the substrate level, starting from a sugar. Theorem 2 states that it is not possible to make more than one ATP from a sugar with less than six carbons. It should be emphasized that these theorems have been derived from a critical hypothesis which imposes a strong thermodynamic condition; a chemical reaction, and particularly a reaction to make ATP at the substrate level, is allowable only if it has an equilibrium constant reasonably high which can account for a high net exergonism. The reason for this hypothesis is the 'metabolic freedom' that provides a reaction with a high ΔG^c, to obtain a high flux and/or a short response time under a wide range of substrate and product concentrations. It must be pointed out that this is a general property in a good metabolic design. In relation to glycolysis, the high equilibrium constant allows the reaction to work even at very high concentrations of products such as ATP and lactate, and low concentrations of substrates (ADP and glucose); in muscle, lactate can reach 100 mM [40].

These theorems are well confirmed empirically since, in many different designs of glycolysis pathways, derivatives of the reaction of Gra3*P* dehydrogenase are involved in all of them to make ATP; from theorem 2, it is concluded that not more than two ATP can be produced by anaerobic metabolism from one glucose, and this result is also in good agreement with most of the glycolysis schemes found in Nature. However, there is an important exception; the glycolysis design of the parasitic Nematode worm *Ascaris lumbricoides* (Fig. 14), whose ATP yield is 3.33/glucose. The reason for this apparent contradiction is not the theorems themselves, but the hypothesis under which they have been proven, as that hypotheses is do not apply there. The two different reactions of *Ascaris* where ATP is made

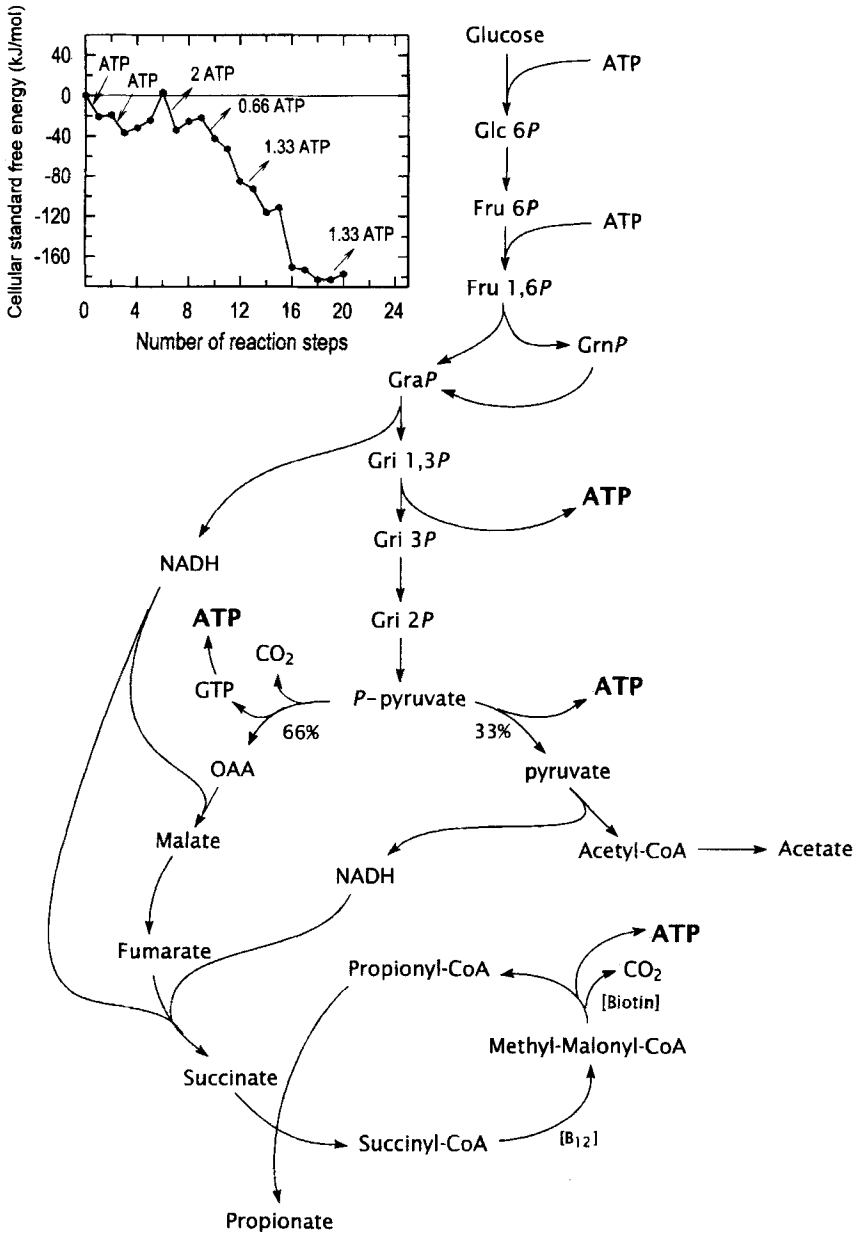


Fig. 14. The *Ascaris lumbriocides* pathway. A route to take ATP from glucose degradation under anaerobic conditions found in this intestinal parasitic Nematode worm [36]. The energetic yield of this route is higher than of the regular glycolysis (3.33 ATP/glucose instead of 2) but it has a very poor design to maximize the flux of ATP production. This route is poor for that purpose, but it is very good for life under a fixed input of material (typical of the parasitic life) where the evolutionary target of optimization is not the flux of ATP production, but its stoichiometric yield. Thus, *Ascaris* glycolysis cannot be considered as another case of paleometabolism, but a quite different design optimized for another purpose.

(*P*-pyruvate carboxykinase, and propionyl-CoA carboxylase, both working in the reverse direction to the regular cell metabolism in other animals), are (isolated) endergonic under cellular standard conditions (Table 4) with a positive cellular standard free energy change. Why cannot this thermodynamic hypothesis be applied in *Ascaris*?

The different target of optimization for the two different constraints mentioned above can well explain this. Maximization of the flux and/or minimization of the response time (the main optimization targets considered) are only possible under conditions of constant substrate concentration, a feature that can only be applied to non-captive animals, which are at liberty to search for food. For instance, prairie grass is virtually unlimited for the antelope. Then, the problem is not particularly the amount of food available, but the physical (metabolic) capability of the animal to use it (to live, in general) safely.

Competition with other individuals of its species is not for food, but to escape from predators, so the flux of ATP production and its rapid acquisition (metabolic response time) is much more important than the stoichiometric energetic yield. Parasitic life operates under other constraints. Here, lack of freedom requires a constant input flux and, therefore, a non-constant concentration of the first substrate (food limited). Consequently, optimization of the flux and a quick metabolic response has no meaning for this kind of life. Our theorems do not apply to parasitic animals because the hypothesis has no meaning there.

In *Ascaris* glycolysis, the stoichiometry has been optimized at the expense of a slow metabolic response. It is important to recognise that this metabolic structure, which depends on a constant input flux, is also designed to maintain a constant steady-state glycolytic activity as a regular ATP source, without a run/

stop pattern. It cannot be stated that the regular glycolysis design present in most species for glucose → lactate conversion, is better than the design in *Ascaris*. The importance lies in the variable to be optimized. The two possible environmental constraints, a constant input or a constant concentration of the first substrate, determine two different optimization targets; if an organism lives under a constant substrate concentration, then it has no need to optimize the energetic yield because there is no restriction on the availability of substrate; the aim is to maximize the flux of ATP production. However, if the substrate is limited at constant input, one must adapt the metabolism to the flux; the energetic yield must be maximized. Such a metabolic structure with a poor capacity for rate change is in good agreement with parasitic life; in a sedentary animal, metabolism must work at constant low rate and a loop of reactions is not important.

Parasitic life is barely mentioned in Darwin's work. Maybe he intuited that somewhat different principles were operating there. In the light of our results, it can be stated that Darwin's principles of natural selection are also of application to parasitic species, but there is a different reason behind the struggle for existence of these two kinds of living beings, which concerns their evolutionary target of metabolic optimization. An antelope in the prairie has a constant (virtually unlimited) amount of grass, assuming that the animal is mobile; a predator is a similar case, as there is abundant prey in the territory which it can embrace; the capability they have to move allows them to cover a large territory; however, a parasite is most like a predator that cannot move to look for sustenance and lives at the expense of food that passes it at a certain regularity. Displacement has practically no meaning for a parasite, as it cannot take more food through it, and so, parasitic animals are not rushed. *Ascaris* can be considered as a paradigm of this kind of life, but here the parasitic condition is used in a broad sense. Other animals living in a reduced space with limited food might also have its metabolism evolved to optimize the energetic yield.

Metabolism of different cells in a multicellular organism may also be optimized for each of these purposes, under these constraints. Thus, red muscle is a good example of a system whose activity depends on a constant input flux of glucose and oxygen through blood circulation; its work rate can only be increased by enhancing the input flux (by increasing the heart activity), and its ATP yield is maximized by coupling glycolysis with the Krebs' cycle and oxidative phosphorylation. White muscle is the opposite case; its activity is independent of blood circulation as its glycolysis substrate is stored as glycogen inside the cell, and its glycolytic activity (anaerobic) is independent of the oxygen supply; therefore, it has its ATP flux maximized to support quick movements. This is also in good agreement with our recent work [19] where we have experimentally demonstrated that glycolysis in white muscle has a shorter metabolic response time than in red muscle. The obvious limit in the capacity of glycogen storage in the cell conditions its capacity to work, but this limit of capacity is for the time to work, not for its power, since the flux of ATP production is maximized. Glycolytic design has a feature which makes it more kinetically efficient; the starting substrate is not glucose but a polymer of it (glycogen) whose synthesis involved two ATP/glucose, and it yields one more ATP in its particular glycolytic design (glycogen → glucose-*P* → lactate). Again, this extra ATP production does not contradict our theorems, since they are derived for monosaccharide metabolism, but it is another good example of an optimized glycolytic design to maximize the flux of ATP production. Red blood cells are another good example of systems living under a constant substrate concentration (5 mM glucose), and they have also maximized the ATP flux, not the ATP yield. This important difference in metabolic organization must have had a repercussion in metabolic regulation. In principle, systems under constant input constraints should be regulated by a 'push' mechanism, while systems under constant substrate availability should be regulated through a 'pull' mechanism. Thus, for example, glycolysis in red and white muscles will be regulated by quite different mechanisms; this is a prediction theoretically derived from this work which requires verification. Also, it can be derived from our results that all the control points of flux in *Ascaris* glycolysis should be limited to the first step of the glucose supply; this also has to be verified.

There is a clear difference from the evolutionary point of view between the different kinds of alternative designs of glycolysis. Entner-Doudoroff (Figs 10, 11) and phosphoketolase (Fig. 12) routes are cases of paleometabolism, less optimized designs. *Ascaris* glycolysis (Fig. 14) is a pathway designed to be adapted for a particular purpose, with a different optimization target. It is composed of pieces of material from other pathways (gluconeogenesis, Krebs' cycle, propionyl-CoA, etc) and cannot be really considered as a primitive design, but rather as a later evolved design. This method of building new metabolism using material previously made for other purposes is an example of evolutionary opportunism working at the molecular level [8, 41].

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