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Optimization of Metabolism: The Evolution of Metabolic Pathways Toward Simplicity Through the Game of the Pentose Phosphate Cycle

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Previous theoretical studies on the pentose phosphate cycle (Meléndez-Hevia *et al.*, 1985, 1988, 1990) demonstrated that simplicity in metabolism, defined as the least possible number of enzyme reactions in a pathway, has been a target in biological evolution. Those results demonstrated that a process of optimization has occurred in the evolution of metabolism. However, the results also suggest a number of questions of general interest: (i) Why simplicity? What is the selective advantage of simplicity in metabolic pathways? (ii) How has simplicity been achieved? Can natural selection mechanisms solve the problems of combinatorial optimization in the design of metabolism? (iii) Are the reaction mechanisms of the pentose phosphate cycle (transketolase and transaldolase) the best suited for pentose–hexose interconversion? For example, could a simpler pathway be possible if other enzymes (e.g. *one* carbon transfer) were to exist? In this paper we analyze all these questions and present results which demonstrate that: (i) Simplicity (the least possible number of steps) in a metabolic pathway is a feature which supplies more catalytic efficiency. That is, for a given metabolic conversion, a short pathway yields more flux than a long one. (ii) Natural selection working at molecular level accounts for the selection of the shortest pathway. (iii) It is not possible to find any other set of enzyme mechanisms capable of producing a simpler solution for the pentose phosphate pathway; any other mechanism, such as one carbon transfer between sugars, leads to a more complicated solution. Therefore, our results demonstrate that both the design of this pathway and the enzyme mechanisms themselves have been optimized.

1. Introduction

Biological evolution is at present understood as a process of optimization capable of accounting for the solution of very complicated problems, natural selection being the only known mechanism for optimization which we know works. It can be considered in fact, that a number of well-demonstrated cases of optimization have already been reported. Surely the first documented case was that of Darwin's famous finches (Darwin, 1839; see also Carson, 1992). Also, a number of cases of convergence, where species of different origin became similar when they occupied similar environments, were reported later. The same result arrived at by different distantly related species when they have to play the same role is obviously a good example of optimization, and it is clear that these examples provide good proofs of biological evolution as well as a demonstration of the machinery of optimization. Nevertheless, cases of optimization

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numerically proven are not abundant. Probably one of the best known is that which concerns the litter-size in birds in accordance with a collection of environmental variables including the limitation of feeding resources and predation. The reasoning behind this was developed by Lack (1947), [see also Owen (1983)], and it has been experimentally demonstrated by Perrins & Moss (1975) by manipulating the litter-size in nests; see also Perrins & Birkhead (1983).

It is clear that any optimization process macroscopically observable must be based on processes of microscopic variable optimization. This obviously relates to cellular metabolism, as the chemical machinery which supports every biological function. Cell metabolism is usually understood as a network of metabolic pathways composed of enzymes catalyzing consecutive reactions through a number of common intermediate metabolites. Although it could be argued that cells do not really have "metabolic pathways" but enzymes, it should also be considered that the pathways are not just a simple abstraction; they have a clear physiological meaning accounting for net fluxes of whole conversions. Any enzyme is, in fact, just a tool of a pathway and thus, the evolution of the "enzyme species" has no meaning without considering the evolution of the metabolic pathways, in the same way that any enzyme has no meaning out of a given chain of transformations.

Modern cellular metabolism is thus the result of a multi-objective optimization process carried out during evolution. Several authors have studied the importance of these objectives, as well as their feasibility, considering a number of them as possible and reasonable targets in the evolutionary process of optimization (see, e.g. Cornish-Bowden, 1976). Heinrich *et al.* (1991) and Heinrich & Hoffman (1991) have summarized the different targets of optimization as follows: (i) maximization of steady-state metabolic fluxes to produce important end products (the main target according to many authors (Waley, 1964; Atkinson, 1969; Cornish-Bowden, 1976; Kacser & Beeby, 1984; Reich, 1985; Dykhuizen *et al.*, 1987; Heinrich *et al.*, 1987, 1991; Heinrich & Hoffmann, 1991; Meléndez-Hevia & Montero, 1991); (ii) minimization of the total concentration of intermediates, and the total osmolarity (Atkinson, 1969; Savageau, 1976; Schuster & Heinrich, 1987; Ovádi, 1991); (iii) minimization of response times, or fast relaxation of metabolic systems to reach stable steady states (Heinrich & Sonntag, 1982; Meléndez-Hevia *et al.*, 1990; Heinrich & Schuster, 1991; Torres *et al.*, 1991); (iv) optimization of controllability (Atkinson, 1969; Heinrich & Holzhütter, 1985; Heinrich *et al.*, 1987; Schuster & Heinrich, 1987), and (v) stoichiometric simplicity (Meléndez-Hevia & Isidoro, 1985; Meléndez-Hevia & Torres, 1988; Meléndez-Hevia, 1990). Also, the conclusions of Reich & Sel'kov (1981) on the elementary properties of energy metabolism of the cell can be considered as targets in the evolutionary process; these include capacity of homeostasis, self-control of input and output rates, threshold and trigger phenomena, autonomous mobility, certain economy of storage, and capability of autocatalysis, among others.

On the other hand, the values of the enzyme parameters have also been changed and selected during evolution in order to achieve an efficient chemical support for the different biological functions. In fact, many of the above-mentioned evolutionary objectives can be attained by achieving an appropriate set of enzyme parameters (particularly the individual rate constants of the reaction). Due to the complexity of any catalytic mechanism, the particular influence of each rate constant on the reaction velocity depends in turn on the values of the rest of the constants, through an equally complicated relationship; therefore, the achievement of appropriate values of enzyme activity is a question of multivariable optimization, which is obviously one of the main bases of evolutionary mechanisms. This problem, studied by Cornish-Bowden (1976), and by Heinrich and his colleagues (Heinrich *et al.*, 1990, 1991; Heinrich & Hoffman, 1991) has a special significance in the evolutionary optimization for designing metabolic pathways, since the physical existance of the pathways (sequential chains of metabolic reactions) is based on the concordance of the activity of their enzymes, which includes their specificity toward appropriate intermediates of the path.

Natural selection was stated by Darwin (1859) as the mechanism through which evolution achieves optimization of these variables. Eigen & Schuster (1979) have given an analytical proof of this by means of hypercycle and quasispecies approaches (see also Küppers, 1983) demonstrating that under conditions of a hereditary selective advantage in only some individuals of the population, and a competition between them, the main group of the individuals eventually acquire a desirable property. Also application of non-equilibrium thermodynamics (Katchalski & Curran, 1965; Nicolis & Prigogine, 1977) to complex biochemical systems has allowed some authors to obtain general conclusions concerning possible evolutionary optimization strategies (Stucki, 1988; Westerhoff & van Dam, 1988; see also Heinrich *et al.*, 1991).

Selection and evolution of the set of cellular enzymes is obviously inseparable from the selection and

evolution of pathways design. Evolution of enzymes (as well as enzyme activity) is usually understood to involve two parts: reaction mechanism and specificity. Nevertheless these two features cannot always be absolutely separated, since certain mechanisms of reaction are only possible with a given specificity, and, conversely, the same basic mechanism necessarily means a different reaction when it is performed on a different substrate. It is generally accepted that the process of enzyme activity optimization carried out in evolution has consisted of the improvement of a given reaction mechanism which could slowly occur (see Kacser & Beeby, 1984) even in an enzyme-free system. Serving as an example are the results obtained by Waddell *et al.* (1987, 1989; Waddell & Miller, 1992), where the same sequences of reactions (glutamic and α-ketoglutaric acid to succinic acid) as occur in modern cells, were carried out in a system free of enzymes or coenzymes, under the influence of sunlight only. Nevertheless, in many cases, a given whole conversion, even based on the same reaction mechanisms, could be achieved by a number of different pathways involving a different number of steps. Thus, since there are different possible ways to obtain a given solution, it can be viewed as a typical problem of combinatorial optimization, whose goals are the searching for a solution with the least possible number of steps. With this view we can envision cellular metabolism "asking" whether its chains of reactions are a good solution for the problem of conversion of material.

2. Remembering the Game of the Pentose Phosphate Cycle

A problem of this kind has been found in cell metabolism by Meléndez-Hevia and coworkers studying the structure of the pentose phosphate cycle (Meléndez-Hevia & Isidoro, 1985; Meléndez-Hevia & Torres, 1988; Meléndez-Hevia, 1990). Cells convert pentoses into hexoses, by means of the pentose phosphate cycle, allowing the conversion of sugars of the informative material (pentoses) into those of energetic and structural material (hexoses). An extensive review of this metabolic pathway can be found in Wood (1985). The non-oxidative phase of this pathway can be formulated as the conversion of six sugars of five carbons each (ribulose 5-phosphate) into five sugars of six carbons each (glucose 6-phosphate) by means of the action of certain enzymes which transfer either two (transketolase) or three (transaldolase or aldolase) carbons from one sugar to another. There are many different ways to achieve this conversion if the specificity of such enzymes is considered not to be strict for particular substrates. Each one of these procedures involves different intermediates and number of steps. The *simplest* path was achieved by means of searching for the best strategy for a game of combinatorial optimization which represents an abstraction of the pentose problem (see Fig. 1). The goal of the game is to find the simplest way for the transformation of sets of five elements into sets of six, according to certain rules expressed as two types of hypotheses: mechanisms (which represent the cellular enzymes, establishing the number of elements which can be transfered among the sets in every step), and simplicity, (which imposes the condition of obtaining the whole conversion in the least number of steps). The mathematical analysis of this problem gives us the best strategy for the game, and this is found to be precisely the way which the pentose phosphate cycle is organized in living cells; in other words, it is mathematically proven that cells have designed their pentose metabolism through the simplest procedure (Meléndez-Hevia & Isidoro, 1985; Meléndez-Hevia & Torres, 1988). The Calvin cycle in photosynthesis has also been analyzed through this approach giving a similar result (Meléndez-Hevia, 1990). The pentose problem is thus a well-established case of optimization achieved by biological evolution, in a certain way very similar to the problem of the littering-size of birds mentioned above. There are, however, some differences between them, namely, the solution of the pentose problem is general for any living cell, while the littering-size is obviously particular for each case; also the solution to the pentose problem is theoretically derived, while the littering-size problem is only empirically demonstrated. In any case, these two examples are numerically stated and stand as two

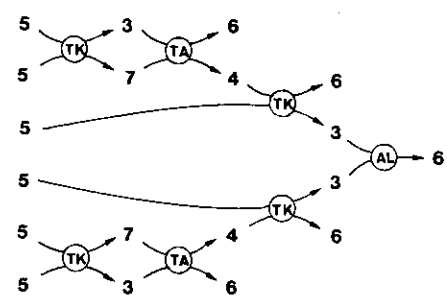


FIG. 1. The game of the pentose phosphate cycle. We have six boxes with five balls each, and must arrive at five boxes with six balls each, in the least possible number of steps, by changing in every step two or three balls from one box to another. This is the simplest solution of the problem, which is similar to the metabolic transformation of six sugars of five carbons into five of six carbons, by means of the transfer in every reaction of two carbons by transketolase (TK) or three carbons by transaldolase (TA) or aldolase (AL). The numbers express the numbers of balls in each box, or of carbons in each sugar. It has been proven that the cell solution is the simplest one (25–27). Thus, the figure also shows the cell pathway for sugar interconversions.

good cases which illustrate that evolution is a process of optimization.

There are two questions which are derived from the hypothesis of simplicity in the pentose problem: (i) why has simplicity been achieved? What is its metabolic meaning? (ii) How has it been achieved? Through which mechanisms have cells achieved it? We analyze these two questions in the first part of this paper. The two kinds of hypotheses (mechanisms and simplicity) are, in fact, quite different, have different meanings, and represent two different features in the process of optimization of metabolism. In the game of pentoses simplicity is the target of the optimization, while the mechanisms are imposed as constraints, assuming they represent the cellular possibilities to reach this objective. However, in spite of the difference between the hypotheses they are closely related since the simplest way to solve these problems is a procedure which could strongly depend on the allowed rules. Thus, a question which arises after the first results on the game of pentoses is: would a simpler solution be possible if the rules of the game were different? Which immediately leads us to ask why the rules of the game (the mechanisms) are precisely the ones observed? Why has evolution selected these enzymes for pentose metabolism? Do they have any particular advantage, or are they there because no other chemical mechanism is possible to interconvert sugars? We analyze these questions in Section 6 and following ones of this work.

3. Simplicity

3.1. DO CELLS NOW PLAY THIS GAME?

The structure of the pathway for these sugar interconversions was first described by Horecker *et al.* (1954), and later widely confirmed (Rognstad *et al.*, 1982; Landau & Wood, 1983; Wood, 1985). Thus, if one observes the cell behaviour in converting pentoses into hexoses, always by the simplest procedure shown in Fig. 1, one must obviously conclude that the cell well knows the optimal solution (it "plays" the game of the pentoses as an expert player). This fact allows us to conclude that this process actually occurs in cells like a well-defined sequential pathway, rather than as a casual intervention of the enzymes looking for a way to carry out the whole conversion. This well-defined chain of reactions is clearly based on the high specificity of the enzymes for fixed substrates, i.e. these "rules of the game" are at the present time very rigid with respect to the substrates of each enzyme. However, it is very reasonable to assume that the more flexible rules mentioned above (see, e.g. Meléndez-Hevia, 1990), were in force in ancestoral cells, when the problem of optimization was not yet resolved, and cells had to find the optimal solution playing the game of the pentose phosphate cycle. Cells no longer play, but it must be accepted that the game has occurred in evolution until the simplest (optimal) solution was found. Cells have become expert players during the evolutionary process. The selection established the substrate specificity, and then, the random set of possible conversions became a well-organized sequential pathway. There are, in fact, so many different possible solutions that the best one can only be achieved by means of very efficient mechanisms of optimization which have obviously operated during evolution. On the other hand, it is clear that the condition of simplicity was observed during this process, which leads us to the following questions: (i) Why has evolution observed this condition, and (ii) How has its attainment been possible? We shall present here results which can answer these questions, showing that the simplest pathway accounts for the greatest flux of metabolic conversions, and that natural selection always leads to select this design.

4. Why Simplicity?

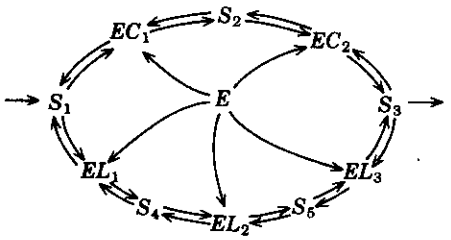
The first question which arises from the considerations presented above is to ask why simplicity has been considered as a condition in the process of pathway design. Although the simplest design always looks more attractive, and it might seem that there are obvious reasons of economy for such a solution, the answer to this question is not trivial. The following theorem gives a good reason for simplicity as well as immediately suggesting the target pursued in the evolutionary process which has led to such solutions.

4.1. THEOREM 1

If a given metabolic conversion is carried out by two alternate pathways which simultaneously operate (the whole flux of conversion being the summation of these two contributions) and which differ in the number of steps (enzyme reactions), with all the enzymes having the same K_m and V_{max}, then the path involving the least number of steps accounts for a greater part of the whole flux.

4.2. PROOF

Chemical affinity (i.e. the negative increases of free energy, which accounts for the electrochemical potential of the reaction) is what determines the tendency of any chemical reaction to occur. Let us consider the system shown in Fig. 2, where an initial substrate S_1 is converted to the end product S_3, through two



$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (ES) \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + P$$

FIG. 2. Hypothetical metabolic system, simpler than the pentose phosphate pathway, but without loss of generality, whose behavior in converting S_1 into S_3 can well illustrate some conclusions of this work. Values of kinetic parameters are as follows: for the short pathway, $k_1 = 3$, $k_2 = 9$, k_{-1} = variable, $k_{-2} = 1/k_{-1}$; for the long pathway, $k_1 = 3$, $k_2 = 3$, k_{-1} = variable, $k_{-2} = 1/k_{-1}$. The value of the equilibrium constant between S_3 and S_1 is 729 (for the two branches of the loop). Variation of the enzyme activities, by modifying its substrate affinities, produces important differences in particular and whole fluxes. Results of a computer simulation of this system upon changes in rate constants are shown in Figs 3(b), 4(a) and 4(b). In order to avoid unnecessary complications in the presentation, all the reactions are unimolecular; this does not involve any restriction on the more general (and realistic) two-substrate mechanism, where the difference among the complexes is clearer.

different pathways, with two and three steps, respectively. (It can be, of course, generalized to any number of steps in each branch, n and m, $n < m$.) The total equilibrium constant between S_1 and S_3 will then be the same, independent of the path which connects these products. Let us also assume that all the steps of the short path have the same K_{eq} values and that all the steps of the long pathway also have the same K_{eq}s (different from the short path K_{eq}s). This leads to the result that the partial chemical affinity of any step of the short path is greater than any step of the long one. These affinities are independent of the reaction mechanisms which connects the two consecutive products [see Fig. 3(a)], because the concentrations of the intermediates depend only on the concentrations of S_1 and S_3, and on the individual equilibrium constants, which are also independent of the reaction mechanism. Let us see now the relationship between the concentrations of these intermediates and the individual reaction affinities. We shall assume first-order kinetics for every reaction, as well as a one-substrate/one-product mechanism, which in no way involves any loss of generality. Thus, any reaction catalyzed by the enzyme E, which converts a substrate S into a product P is described by:

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} P + E. \qquad (1)$$

Provided that the free-enzyme concentration is constant, its value can be introduced within the kinetic constants k_1 and k_{-1}:

$$S \underset{E \cdot k_{-1}}{\overset{E \cdot k_1}{\rightleftarrows}} P \quad \text{or} \quad S \underset{k'_{-1}}{\overset{k'_1}{\rightleftarrows}} P. \qquad (2)$$

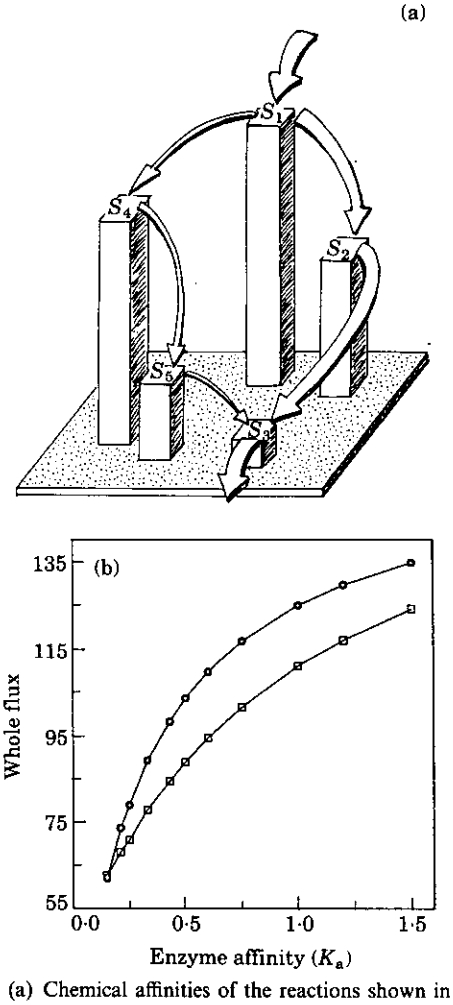


FIG. 3. (a) Chemical affinities of the reactions shown in Fig. 2. Bars represent the chemical potentials (μi) of the intermediates. The height differences between the bars represent the chemical affinities. Note that the chemical potential of the last intermediate of the short pathway (S_2) is greater than the last one of the long pathway (S_5), which accounts for a greater flux through the short branch. (b) Effect of the activation of each pathway on the whole flux when there is no limit to the total amount of enzyme. The metabolic system shown in Fig. 2 converts S_1 into S_3 through two alternate pathways. Under conditions of constant free enzyme (no limit to the total amount of enzyme) the increase of activity of the enzyme for the short or the long pathway, by means of increasing its affinity for their specific substrates, always promotes an increase of the whole flux, but the effect on the short pathway is greater than that on the long one. Initial conditions were: short pathway, $k_1 = 3$, $k_{-1} = 20$, $k_2 = 9$, $k_{-2} = 0{\cdot}05$; long pathway: $k_1 = 3$, $k_{-1} = 20$, $k_2 = 3$, $k_{-2} = 0{\cdot}05$; Enzyme affinity, plotted in the abscisa axis, is defined as $K_a = k_1/k_{-1}$; this was changed by varying k_{-1}, where $k_{-2} = 1/k_{-1}$. Amount of free enzyme (constant) was $e = 10$; other data are described in Fig. 2. Whole flux, plotted in the ordinate axis, is the summation of the flows which circulate through the two branches of the ringlet. Specific contributions of each pathway to the whole flux are not shown.

Moreover, the reaction rate can be expressed as a function of the reaction chemical affinity A:

$$v = k_1' \cdot S \cdot (1 - e^{-A/RT}). \quad (3)$$

Seeing eqn (3), it is clear that if two reactions have the same value of k_1', the one that has A greater also has v greater. Since the chemical affinity of every step of the short pathway is greater than that of the long one, it follows that the flux through the short pathway is also higher. Also, it is clear that this property can be easily generalized for n pathways, all of them with a different number of steps. In effect, the pathway which contributes the greatest flux is found by the following procedures: comparing one of these pathways with any other one leads to discard the long one, since the short one has more flux; then, repeating the procedure successively will lead us to the shortest pathway.

The selective advantage of the short pathway can be well understood by this property, as well as by the following corollary derived from it. *Corollary*: under the same conditions of the above theorem, a given increase of activity (by increasing the affinity for their substrates) in the enzymes of the short pathway, causes a higher increase of flux than that produced by a similar increase of activity in the enzymes of the long pathway. This property is easily derived from the above theorem by taking derivatives in eqn (3). Let v_C be the flux through the short pathway, v_L the flux through the long one, and v_T the total flux ($v_T = v_C + v_L$); eqn (3) gives the expression for v_C and v_L. Let k_C and k_L be the "k_1'" rate constants of the short and the long pathway, respectively. Regarding eqn (3) it can be seen that $dv_T/dk_C > dv_T/dk_L$. Figure 3(b) shows the effect of a series of activations on the short and long pathway, respectively, in the system represented in Fig. 2. This behavior illustrates well the properties presented here. Furthermore, the property previously demonstrated by Heinrich *et al.* (1987) can be derived—that the value of the maximum possible flux which a metabolic system can get in an optimization process decreases as the number of steps of the reaction chain increases. Moreover, we must mention here the result obtained by Savageau (1975, 1976) which shows another selective value of stoichiometric simplicity: the reduction of the number of steps in a metabolic pathway contributes to the achievement of stable steady states.

However, neither the theorem nor its corollary necessarily explain how evolution always achieves the simplest design of a pathway. In effect, one could think that under the conditions assumed above, a mutation could promote a certain increase in the enzyme activity of the long pathway, without affecting the short one, and it might drive most of the whole flux through this path, which would favour its selection, once it has achieved a selective advantage. This could have occurred accidentally, since the optimization process is long and rough (we will discuss it below), but this could never have been an important obstacle in the process of optimization which leads to the simplest pathway. (In fact, regarding again the structure of the pentose phosphate and Calvin cycles, it is clear that no opportunity seems to have been given to chance, and that precisely the best solution has been accurately selected.) In effect, the selection of the simplest pathway is inevitably assured, assuming that total freedom exists for any mutation to occur either activating the short or the long pathway. We shall demonstrate now that under reasonable cellular conditions, only the mutations that enhance the activity of the short pathway are able to be incorporated into the system (which leads to its selection), while the mutations that enhance the activity of the long pathway are discarded (which obviously leads to avoid the improvement, and consequent selection, of the long pathway).

It is accepted that a number of mutations occurred on the enzymes which accounted for an early set of possible reactions, determining their specificity (and consequently the sequence of reactions, i.e. the structure of the pathway). The effect of such changes on the whole flux is dramatic in a system which contains the following features: (i) the same enzymes—provided that they do not have a very strict specificity—intervene in the catalysis of the alternate pathways; (ii) the total amount of enzyme is fixed; and (iii) the different enzyme affinities for the different substrates (which are precisely the specific intermediates of every pathway) determine the enzyme distribution among the substrates. This set of hypotheses well describes a reasonable realistic model of the evolutionary conditions of metabolic pathways. Now it can be seen that all the features here presented place the evolution of metabolic pathways under strictly Darwinian conditions.

5. Competition Leads to Simplicity

Darwin's theory of natural selection clearly imposes the condition that a competition must exist between two systems to guarantee that the feature which can improve the species is selected; analytical proof has been supplied for this assertion (Eigen & Schuster, 1979; Küppers, 1983). It can be observed that the set of conditions shown above, whose occurrence during evolution is well justified, is precisely that set which guarantees the selection of the pathway

which gives the greatest flux. The reason for this fact can be expressed by the following property. Assume the same conditions as in the above theorem, but with the *same* enzymes catalyzing the reactions involved in the two pathways, and the total amount of enzyme fixed. Then, the function which relates the whole flux with the affinity of the enzyme for each specific substrate of the two pathways has its greatest maximum where the values of enzyme affinity are zero for the substrates of the long pathway and different from zero (positive) for the short one. In fact, it can be seen that this property describes a particular case of Darwinian natural selection. In any case, it can be easily understood taking into account that (i) the short pathway is more effective, as has been demon-

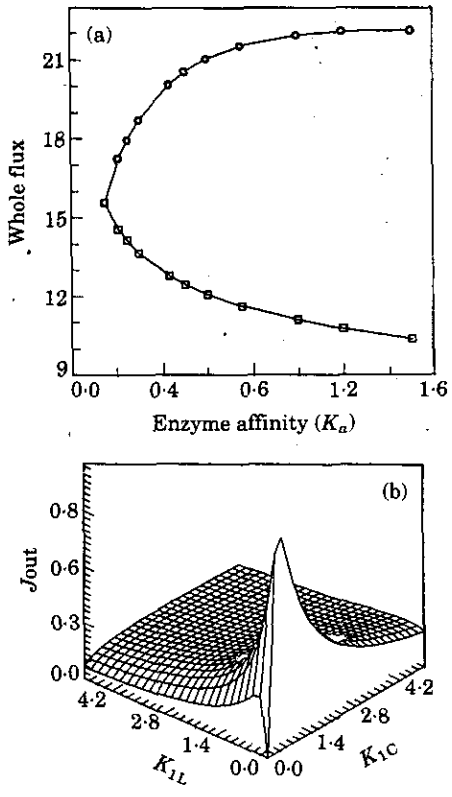


FIG. 4. (a) Effect of the activation of each pathway on the whole flux when the total amount of enzyme is limited. On the same system described in Fig. 2, the same variations in the enzyme activities described in Fig. 3(b) were carried out, with the only difference that here the total amount of enzyme was fixed ($e_T = 10$). Note the difference between this behavior and that shown in Fig. 3(b). Here, activation of each pathway (by increasing the enzyme affinity for its substrates) always decreases the concentration of free enzyme, thus damaging the other branch, since there is less enzyme available for its complexes. This effect of competition (impossible when free enzyme is constant) leads to different results, in accordance with the pathway which is activated conditioning the selection of the simplest pathway. Note that activation of the short pathway always promotes an increase in the whole flux, while activation of the long one leads to a net decrease of it, since the damage that activation of the short pathway produces on the long one is entirely compensated by the increase in the flux promoted by such an activation. However, the damage in the short pathway which is produced by the activation of the long one cannot be compensated. (b) Tri-dimensional representation of whole flux vs. k_{1L} and k_{1C} of the system shown in Fig. 2, in which the total amount of enzyme is kept constant. Effect of different increases of activity (by increasing the affinity of the enzyme for respective substrates) in the two alternative pathways of the system shown in Fig. 2 on the whole flux of conversion of S_1 into S_3. The total equilibrium constant, as well as the equilibrium constant of each step were taken as 1. The following kinetic constant values were employed: $k_{1C} = k_{-2C} = k_{-1L} = k_{-2L} = 1$; $k_{1C} \cdot k_{2C} = k_{1L} \cdot k_{2L} = 1$. S_1 and S_3 were taken as 10 and 1, respectively. As can be observed in this figure, though local maxima appear, the global maximum corresponds to a null affinity of the enzyme for substrates of the long pathway.

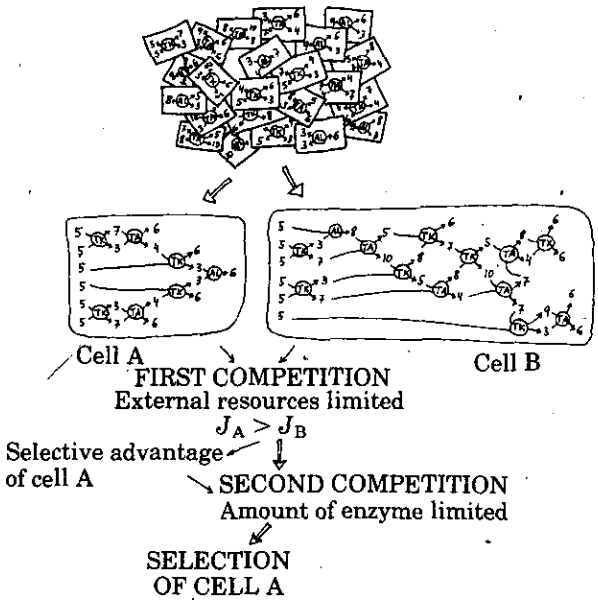


FIG. 5. Schematic summary of the process of optimization in the design of metabolic pathways. In a first stage a set of enzymes without strict specificity can catalyze many different reactions for interconversion of sugars (see the meaning of the enzyme abbreviations in Fig. 1). The different random combinations among these reactions can give many different solutions to the problem of converting pentoses into hexoses. Certain mutations define the affinity of these enzymes, the affinities being different in different populations. These different specificities condition different structures of the pathway to convert sugars, one of them being shorter than the other one. Then, the pressure of two different competitions imposes the selection of the short one. The first is an external competition based on the limitation of resources (e.g. food); this defines the selective advantage of the short pathway, because it supplies more flux and is therefore more efficient for its particular purpose. This competition is established among every group where different pathway designs have been organized, always giving selective advantage to the simplest one. Now, the different mutations on enzyme activity can improve the efficiency of each pathway, but the selection of the simplest one is guaranteed by the second competition. This is an internal competition, which occurs inside the cells, and is based on the fact that the total amount of enzyme is limited; it leads to a general increase of the whole flux when the short pathway is increased (the value of the function in Fig. 2 approaches the maximum) while the improvement of the long one moves the system away from the maximum. So, the pathway which uses the same quantity of enzyme more efficiently is inevitably selected.

strated; (ii) any given increase of the enzyme affinity for the substrates of any pathway always leads to a certain decrease of the free enzyme, and (iii) the short pathway uses less amount of enzyme, since it involves fewer steps, and therefore fewer complexes. The behaviour of the system shown in Fig. 2 under these conditions is shown in Fig. 4. The same variations of enzyme affinity for the substrates that accounted for the behavior shown in Fig. 3(b) produce an effect quite different when the same enzyme catalyzes the two pathways, and the total amount of enzyme is fixed, as can be seen in Fig. 4(a). A tri-dimensional plot of the whole flux function with respect to the two enzyme affinities (for the short and long pathways, respectively) is shown in Fig. 4(b). Note that the unique maximum in the flux function occurs at a zero value for the affinity of the enzyme for the long pathway. This means that any mutation improving the activity of the long pathway will inevitably be discarded, thereby always eliminating such a pathway and leading to the selection of the short one.

It is necessary to point out that these results are consequences of the same enzyme accounting for a number of different conversions, and that the total amount of such an enzyme is limited, two conditions which are perfectly applicable in the evolution of metabolic pathways. When the same enzyme catalyzes reactions of two different pathways the activity of each path of a ringlet such as shown in Fig. 2 is not independent of the other one. The enhancement of affinity of the enzyme for the different substrates of a given pathway increases the fraction of enzyme to form the corresponding complexes, leaving less enzyme available for the other ones, thus, damaging their pathway. A competition is then originated between the different substrates (and pathways) for the same enzymes, emphasizing the selective advantage of the simplest pathway (which spends less enzyme and is more efficient) against the other one, when the flux is the variable to be optimized. This is illustrated in Fig. 5. This competition leads to natural selection operating on given changes in the specificity of the enzymes for the different substrates (which determine the pathway to be selected). It leads to the selection of the simplest pathway. *Simplicity is, therefore, a consequence of the evolutionary conditions, more than a hypothesis arbitrarily imposed in the model.* The design of the pentose phosphate cycle in cells, which is the simplest solution of the problem (the best strategy of the game) is, therefore, the result of an optimization process. During this process the rules of the game with respect to substrate specificity were adjusted in order to achieve the shortest chain of reactions for the whole conversion. It is not by chance that cells have found the simplest solution to the pentose problem. (They have learned the best strategy of the game.) Evolution of metabolic mechanisms has inevitably led in this direction. The pentose phosphate cycle (as well as the Calvin cycle) is, therefore, a paradigm of metabolic optimization, and possibly the general ideas derived from this study can also be applied to other metabolic pathways.

6. Enzyme Reaction Mechanisms

6.1. THE REASONING BEHIND THE RULES

Once simplicity has been explained we must realize that it has been stated under a given set of rules (reaction mechanisms) according to the available cellular enzymes. However, observing metabolism one could think that these mechanisms might have been arbitrarily selected, and that maybe under a different set of rules the present best solution could be improved upon. Therefore, the next step in this research should be to explain the reason for these hypotheses, i.e. why the cell has precisely these enzymatic mechanisms instead of other ones. Why do the characteristic enzymes of the pentose phosphate path-

TABLE 1

Structural features of the sugars which participate in the reactions catalyzed by cellular transketolase and transaldolase, typical examples of two substrates/two products transferases with a ping-pong kinetic mechanism. These enzymes transfer one 2- *or* 3-*carbon unit between two sugars. In all of them the donor ketose becomes aldose, and the acceptor aldose becomes ketose*

Carbon unit transferred	Transketolase C_2		Transaldolase C_3	
	Donor	Acceptor	Donor	Acceptor
Functionality	Ketose	Aldose	Ketose	Aldose
No. of carbons	C_5, C_6, C_7	C_3, C_4, C_5	C_6, C_7	C_3, C_4
Chirality	C-3 (*S*)	—	C-3 (*S*)	—

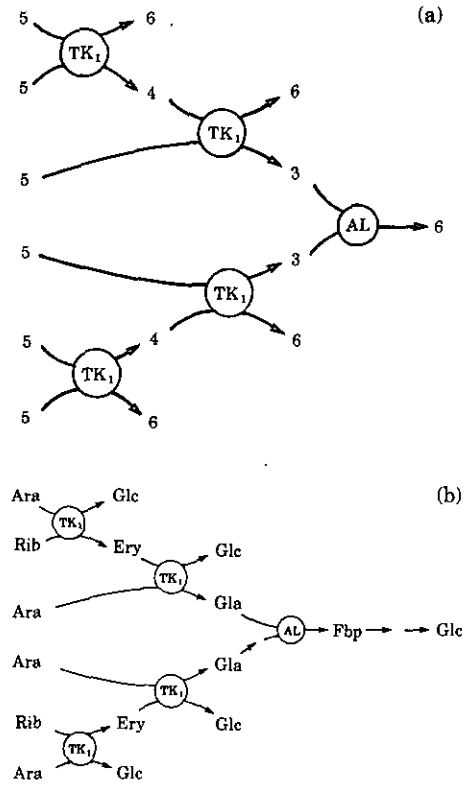


FIG. 6. A possible route for converting pentoses into hexoses which looks reasonable, and is apparently simpler than the cellular pathway. This route is based on a one-carbon transfer mechanism. This mechanism is chemically feasible, but whole pathway (b) is not (see the text). Only reactions involving transfer of carbon are shown: TK_1, an hypothetical transketolase which would transfer a one-carbon unit; AL, aldolase. (a) Basic scheme showing only the number of carbons of the intermediates. (b) Case 1, one possible pathway where transketolase would be specific for aldose donors with their C-2 in R configuration.

way (transketolase and transaldolase) operate under these particular mechanisms? (see Table 1). Could the cell have different enzymes which would be able to account for a simpler pathway? This question can be more explicitly asked: why could there not exist a mechanism of one-carbon transfer? It can be observed that if a mechanism of one-carbon transfer is assumed, a solution simpler than that shown in Fig. 1 seems possible (see Fig. 6). We shall determine now if this solution is possible. It involves two kinds of analysis: (i) a chemical analysis in order to see if there is any chemical feature which does not allow such a mechanism, and (ii) a mathematical analysis, in order to see whether such a solution, if chemically possible, is consistent with all other rules. We shall demonstrate here that this solution, which apparently is simpler than that which occurs in cellular metabolism, is not possible, and that any other solution simpler than the cellular one cannot exist, even by changing the rules of the enzyme mechanisms.

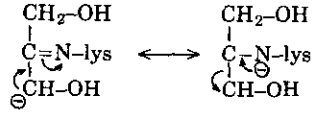
The first feature that is observed in Fig. 6(a) is that the sequence (555)→(654)→(663) in two steps is irreducible whatever the number of carbons transferred in each step, since only two sugars can participate in each step and we have to convert three; it is also the simplest possible one whatever the number of carbons transferred in each step. There are, of course other solutions with mechanisms of one-carbon transfer, different from that shown in Fig. 6(b), each involving different intermediate sugars (although with the same number of carbons). We will show that in no way do they alter the results obtained with the analysis of Fig. 6. This hypothetical solution is based on an enzymatic mechanism to transfer a one-carbon unit between two aldo-sugars, provided that the donor has its second carbon in R-configuration. We do not assume any particular mechanism for these reactions (such as a like-transketolase or a like-transaldolase, or other possibilities available to living cells). Also, all structural features of the sugars are taken into consideration, because they are necessary to determine the possibilities of the enzyme specificity.

7. Chemical Analysis

We will begin with a chemical analysis of the problem. The question is: Is the transfer of one-carbon unit between sugars *chemically* possible? We analyze the possibilities of one-carbon transfer among sugars, which includes the transketolose (TK) reaction based on thiamin pyrophosphate (TPP) catalysis, transaldolase (TA), and tetrahydrofolate (THF)-like mechanisms. We shall demonstrate that transfer of a one carbon unit between sugars is a reaction perfectly possible, even using cellular coenzymes catalyzing well known reaction mechanisms. Let us consider possible mechanisms.

7.1. TRANSALDOLASE

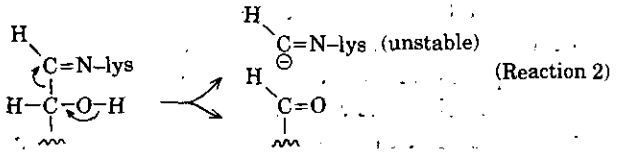
Is a transaldolase C_1 transfer (TA_1) possible in sugars? The modern transaldolase enzyme (TA_3) transfers a C_3 unit from a donor ketose to an acceptor aldose, as a resonance-stabilized dihydroxyacetone anion (lysine Schiff base).



(Reaction 1)

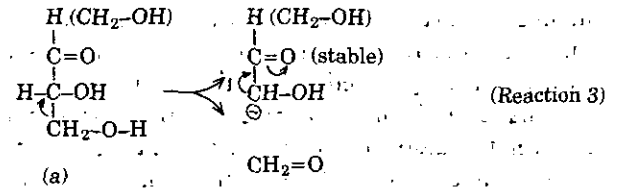
By comparison, an analogous TA_1 process (illustrated below) would not directly yield a stabil

ized anion and is not chemically possible for that reason:



(Reaction 2)

The aldol-like mechanism shown in Reaction 3 could be allowable for sugars of the structural type a.



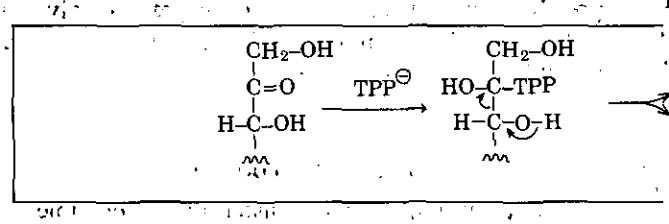
(Reaction 3)

But note that (a) would have to be specifically glyceraldehyde, a 2-ketotetrose, or a 3-keto sugar. Thus, it is clear that such a mechanism does not solve anything, because in the pathway that we are considering we would need TA even for the first reaction, with two pentoses as substrates. Moreover, if we consider that these sugars are phosphorylated (at C-3 or C-4), this reaction is not possible.

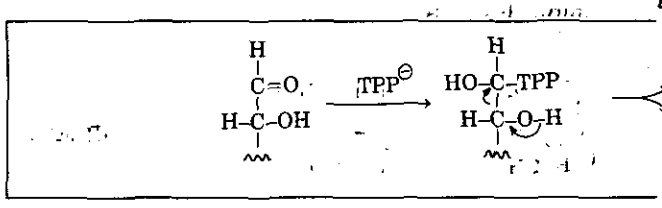
7.2. TETRAHYDROFOLATE

Would a C_1 transfer using the coenzyme tetrahydrofolate (THF) be possible between sugar molecules? There are many examples in modern metabolism of THF transferring a C_1 unit as the chemical equivalent of

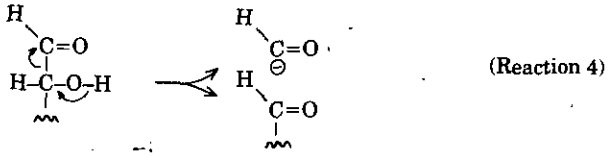
$$CH_3\oplus,\ CH_2\oplus\oplus,\ \text{or}\ H-\overset{\oplus}{C}=O$$



(see e.g. Metzler, 1977). Can THF serve to carry these C_1 entities in monosaccharides? The answer is *no*. Let us examine the different possibilities.

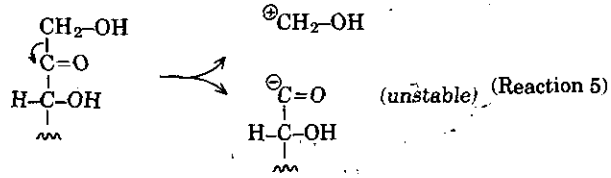


(i) If an aldose is the donor for this C_1 transfer (see Reaction 4) the unit expelled is an unstable acyl *anion*, and this is not compatible with the cation units carried by THF.



(Reaction 4)

(ii) If a 2-ketose is the donor (see Reaction 5) it yields a $CH_2\oplus\oplus$ equivalent, which could be transported by THF, but the other product of this process is an unstable anion. This acyl anion would require a second coenzyme (like TPP) to stabilize it.

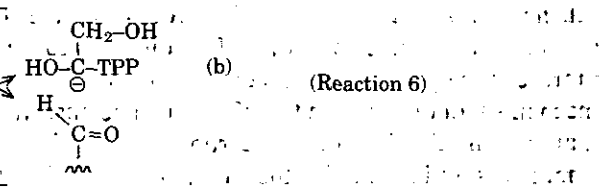


(Reaction 5)

(iii) The cleavage of sugar (a) in Reaction 3 (considered above as a TA_1 possibility) can also be viewed as a $CH_2\oplus\oplus$ transfer. However, the same reasoning carried out there leads us to discard this mechanism with THF; this reaction is not possible between pentoses. Thus, consideration of the possibilities of THF leads us to eliminate a THF C_1 mechanism as a simplifying solution to pentose phosphate pathway reactions.

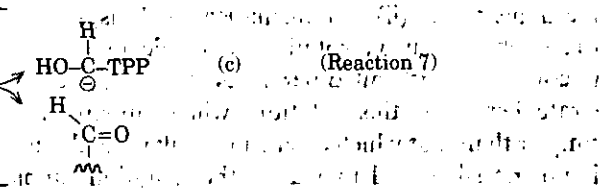
7.3. IS A TRANSKETOLASE C_1 TRANSFER (TK_1) POSSIBLE IN SUGARS?

We shall show that the answer is *yes*, this mechanism being as feasible as the familiar TK_2. The modern cellular TK reaction (TK_2) involves the coenzyme TPP whose role is to stabilize an otherwise unstable acyl carbanion derived from a 2-ketose sugar (see Reaction 6).



(Reaction 6)

Hypothetical TK_1 would have to operate on an aldose, in order to give an analogous C_1 unit. Applying these bio-organic principles for this mechanism we get Reaction 7:



(Reaction 7)

Reaction 6 is absolutely equivalent to 7, and there is no obvious reason why anion (b) and (c) should not

be of approximately equal stability and form from the sugar in the same manner. It is clear that cellular TK_2 catalyzes Reaction 6 by reasons of active site specificity, but not because of chemical restrictions. In other words, selection of TK_2 mechanism versus TK_1 has been carried out not for chemical reasons. This conclusion is well supported by the following facts:

(i) The TK_1 (C_1) intermediate has been experimentally demonstrated by Krampitz and coworkers (Krampitz *et al.* 1962; Krampitz & Votaw, 1966; Krampitz, 1969) studying catalytic functions of thimin pyrophosphate with formaldehyde as substrate in the acetoin condensation to give glycolaldehyde, where the TK_1 anion must be involved (Reaction 8).

$$H_2C{=}O + TPP \xrightarrow{pH\ 8{\cdot}5} \underset{\text{Dihydroxyethyl–TPP}}{CH_2(OH)–CH(OH)–TPP} \qquad \text{(Reaction 8)}$$

(ii) Uhleman & Schellenberger (1976), (see also Kluger, 1987) found that (TPP)-pyruvate decarboxylase catalyzes the cleavage of glyoxylic acid to give carbon dioxide and the

$$HO—\underset{\ominus}{C}H—TPP$$

species (TK_1 intermediate in our reasoning).

(iii) Furthermore, in collaboration with this work, J. E. Bartmess (personal communication, 1990) has carried out calculations (MNDO program)

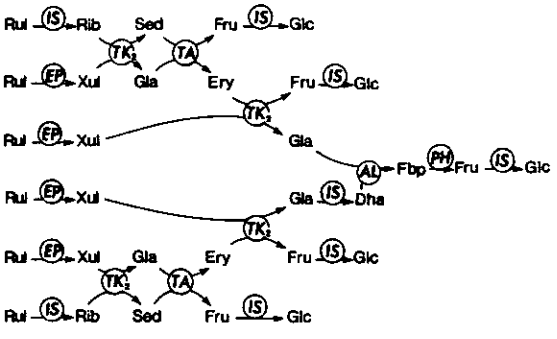


FIG. 7. Scheme which shows the complete stoichiometry of the non-oxidative phase of the pentose phosphate cycle as it occurs in living cells. In contrast to the schemes of Figs 6 and 8, this one is based on a transketolase which transfers a two-carbon unit (TK_2). Other enzymes which participate in carbon transfer are transaldolase (TA) and aldolase (AL). This scheme also includes the "auxiliary" enzymes necessary to convert the initial substrate (ribulose 5-P) into the appropriate substrates of TK_2, as well as the resulting hexoses into glucose 6-P: IS, isomerases; EP, epimerase; PH, phosphatase. Abbreviations of sugars: Dha, dihydroxyacetone phosphate; Ery, erythrose 4-P; Fbp, fructose 1,6-bis-phosphate; Fru, fructose 6-P; Gla, glyceraldehyde 3-P; Glc, glucose 6-P; Rib, ribose 5′-P; Rul, ribulose 5-P; Sed, sedoheptulose 7-P; Xul, xylulose 5-P.

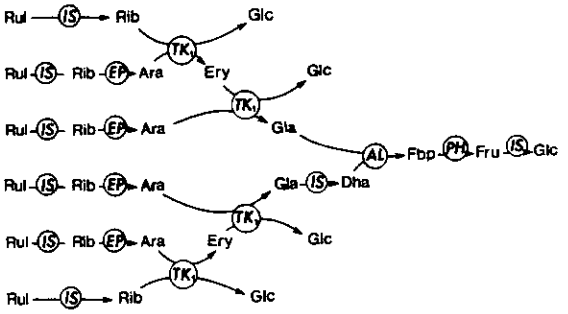


FIG. 8. Scheme which shows the complete stoichiometry of a hypothetical pathway for converting pentoses into hexoses by means TK_1 mechanism. This is the same mechanism as shown in Fig. 6(b), but including all "auxiliary" reactions of isomerases, epimerases and phosphatase, to convert ribulose 5-P into glucose 6-P. This pathway has 18 steps, while the cellular one has 20 (see Fig. 7). Thus, it looks simpler. However, it has been proven to be physically impossible (see the text). All abreviations are the same as in Fig. 7 plus: TK_1, transketolase which transfers a one-carbon unit; Ara, arabinose 5-P.

on (b) and (c) analogs which indicated that they are of approximately the same stability.

$$\underset{\text{(b) } TK_2 \text{ intermediate}}{HO–\underset{\ominus}{C}(CH_2–OH)–thiamin} \qquad \underset{\text{(c) } TK_1 \text{ intermediate}}{HO–\underset{\ominus}{C}(H)–thiamin}$$

Thus, we conclude that the TK_1 process is chemically allowable, and that the reason that modern cells have selected TK_2 in the pentose phosphate pathway is *not* based on a fundamental chemical difficulty with the TK_1 step, but on other reasons.

At this point, once the chemical possibility of a TK_1 reaction has been stated, the problem becomes more interesting. If no chemical possibility were to exist for one-carbon transfer between sugars then the answer to this question would be trivial because no opportunity would have been given to natural selection for checking this possibility. Why was TK_1 *not* selected as a mechanism in the pentose phosphate pathway?

Let us now examine which pathways could be designed using TK_1 in accordance with the two possible specificities required for such an enzyme.

Case 1

The sugar (aldose) which donates the C_1 unit has its C-2 in *R* configuration, and has no particular specificity for the chirality at C-2 of the acceptor; this leads to the pathway shown in Fig. 6(b): two reactions convert three pentoses into two hexoses plus one triose by (555)→(645)→(636).

Case 2

The sugar (aldose) which donates the C_1 unit has its C-2 in *S* configuration, and has no particular

TABLE 2

Reactions catalyzed by the hypothetical enzyme TK_1. Only Reactions 1 and 2 are included in the pathway of Fig. 8, but Reactions 3 and 4 should also occur if 1 and 2 do. The pathway shown in Fig. 8 is, thus, not possible. All donor sugars are aldoses, and have their C-2 in R configuration

	Donor		Acceptor	Acceptor		Donor
(1)	Ribose	+	Arabinose	↔ Erythrose	+	Glucose
(2)	Erythrose	+	Arabinose	↔ Glyceraldehyde	+	Glucose
(3)	Ribose	+	Ribose	↔ Erythrose	+	Allose
(4)	Erythrose	+	Ribose	↔ Glyceraldehyde	+	Allose
(5)	Ribose	+	Glyceraldehyde	↔ Erythrose	+	Erythrose
(6)	Glucose	+	Ribose	↔ Arabinose	+	Allose

specificity for the chirality at C-2 of the acceptor; this leads to a similar pathway (not shown).

However, application of these procedures for converting ribulose 5-P into glucose 6-P leads to different results, because different isomerization and epimerization reactions are necessary to convert ribulose into the appropriate pentoses, as well as the hexoses of these pathways into glucose. Under these constraints Case 2 has no interest, since it generates a pathway with 26 steps (results not shown, but easily derived), more complicated than the cellular one (Fig. 7) which has 20 steps. However Case 1 generates a pathway (see Fig. 8) apparently simpler than cell one, with 18 steps. The question remains: if use of TK_1 leads to a simpler solution to the problem, why has TK_2 been selected instead of TK_1? We shall answer this question, demonstrating that such a mechanism [Figs 6(b) and 8] is not possible. The analysis of the possible specificities of TK_1 has allowed us to arrive at this conclusion, as can be seen in the next section.

8. The Limit of Specificity of Enzymes

The analysis of this hypothetical TK_1 will give us the following conclusion: the specficity of a given enzyme cannot be as strict as one wants; there is a limit in diminishing the range of specificity of a given enzyme on a series of possible substrates. This limit is not empirical, but theoretical, so it is impossible to exceed, and depends on the kind of reaction that the enzyme catalyzes. Let us analyze the TK_1 case; this will demonstrate its limit of specificity, and will lead us to the conclusion that the pathway shown in Fig. 8 cannot occur under any condition, whatever the mechanism of carbon transfer. This property is expressed by the following theorem which states the limit of specificity:

8.1. THEOREM 2

If an enzyme which catalyzes the Reactions 1 and 2 in Table 2 exists, then this same enzyme also catalyzes the reactions (3), (4), (5) and (6) of this Table.

8.2.1. *Proof*

The reasoning is based on the two following accepted principles.

(i) *The principle of activity* (Meléndez-Hevia, 1990). If a given enzyme with activity exists, and its substrates are available, this enzyme will catalyze its reaction.

(ii) *The principle of microscopic reversibility* (see Tolman, 1938; Gould, 1959). Taking into account only the chemical mechanism of the reaction (i.e. leaving out of consideration thermodynamic or inhibitory features), if a given enzyme catalyzes a certain reaction, then this same enzyme will also catalyze the reverse reaction through the same mechanism.

We shall prove this theorem by trying to reduce the range of specificity of this enzyme to only one of the Reactions 1 and 2 leaving the rest out. It will be demonstrated that it is not possible, i.e. that any set of conditions which allow the enzyme to catalyze reactions (1) or (2) also requires that it catalyzes all the other four, and that any additional limitation of specificity renders this enzyme incapable of catalyzing both reactions (1) and (2). The reasoning is as follows:

Let us consider Reaction 1, ribose + arabinose ↔ erythrose + glucose (see Fig. 9); the activity of this enzyme is the transference of one-carbon unit from an aldo-sugar to another. Therefore, and in accordance with the principle of reversibility, the four sugars (two substrates and two products) which intervene in the reaction are aldoses; note that all of the sugars shown in Table 2 are aldoses. Now, in addition to this

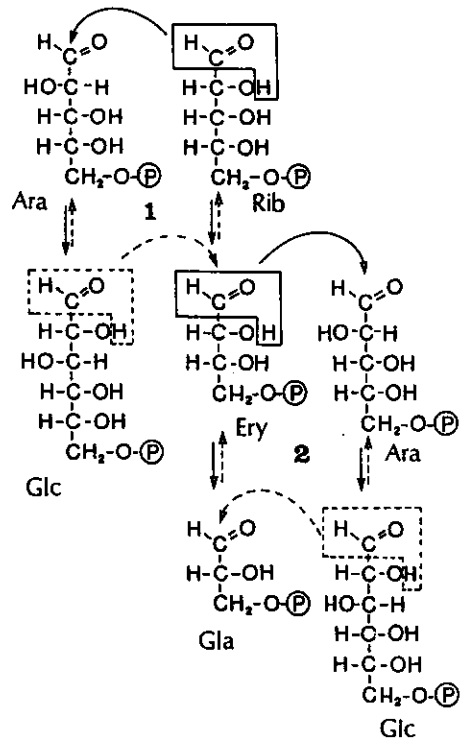


FIG. 9. Reactions 1 and 2 of Table 2, and TK_1 reaction scheme. A formaldehyde group taken from the first carbon of an aldose donor (C_n) with its C-2 in *R* configuration is transferred to the C-1 of the aldose acceptor (C_m). So the donor becomes C_{n-1}, and the acceptor C_{m+1}, both aldoses. Transferred groups are square-marked. Forward directions of both reactions are marked by continuous lines and arrows; reverse directions by broken lines and arrows. Note that donors always have C-2 in *R* configuration whatever the reaction or direction, but C-2 of acceptors is *R* in some cases and *S* in others.

feature, we must assume that this enzyme (defined by its ability to catalyze Reaction 1) should have some feature to determine a certain specificity for its substrates. Otherwise, it would catalyze any other reaction, among any aldoses, which also would include 2, 3, 4, 5 and 6.

The only way to reduce the range of specificity of the enzyme is to impose certain restrictions on the structure of its possible substrates. These restrictions can involve the number of carbons and/or carbon configuration; let us first consider carbon configuration. Regarding Reaction 1 in the two possible directions (see Fig. 9), it can be seen that with the exception of an R configuration for C-2 of the donor, no other structural restriction can be imposed for the configuration of other carbons in the donor or in the acceptor. In effect, in the same reaction, C-3 of the donor is R in ribose and S in glucose; thus, if we impose, for example, a R configuration for C-3 of the donor, this condition is only accomplished by ribose, on one side of the reaction, but not for glucose on the other side; so this restriction would infringe upon the principle of reversibility. Let us now consider whether a C-2 configuration of the acceptor can admit any constraint: if it must be S, it is accounted for in Reaction 1 by arabinose, but not by erythrose, which again infringes upon the principle of reversibility, because the enzyme could only catalyze one of the two directions of the reaction. (Since the process of the reaction demands that C-3 of the donor aldose becomes C-2 of its aldose product which is the acceptor in the opposite direction, the same restrictions apply to the reverse reaction.) A similar reasoning can be applied to Reaction 2. Finally, any additional constraint involving configuration of other carbons, both in the donor or in the acceptor, does not reduce the set of reactions of Table 2, since they are R for all the sugars. Therefore, any constraint which was intended to add to this enzyme, involving the structural features of its substrates, is not possible. So, any enzyme capable of catalyzing Reaction 1 can also catalyze Reaction 2. Now, note that all the sugars involved in the other reactions shown in Table 2 also have the same structural features which means that this

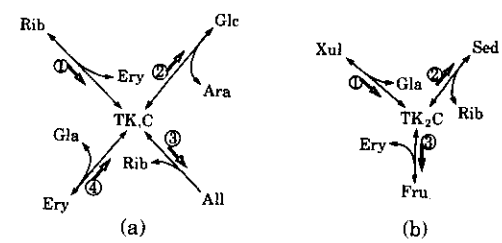


FIG. 10. Scheme showing all the possible reactions catalyzed by the two transketolases studied in this work: (a) hypothetical transketolase (TK_1) which transfers a one-carbon unit from an aldose to another; the donor must have its C-2 in *R* configuration and can have four, five or six carbons; the acceptor can have three, four or five carbons. (b) cellular transketolase (TK_2) which transfers a two-carbon unit from a ketose to an aldose; the donor ketose must have its C-3 in *S* configuration, and can have five, six or seven carbons; the acceptor aldose can have three, four or five carbons. These reactions are explained by the following example (xylulose + ribose ↔ glyceraldehyde + sedoheptulose): TK_2 takes a two-carbon unit from xylulose giving glyceraldehyde and the complex TK_2C; TK_2C then transfer the two-carbon unit to ribose giving sedoheptulose. Any combination of two-forked arrows gives a possible reaction catalyzed by each enzyme, since all these half reactions are reversible; there are three possible combinations for TK_2, and six for TK_1 (see Table 2). However, the thermodynamic condition of a net flux of conversion of pentoses into hexoses determines a given direction in each half-reaction, making some combinations not possible (e.g. for TK_1, 1–2, is possible, but 1–4, and 2–3 are not). The directions which operate are also indicated in the scheme. They are theoretically derived, and furthermore were obtained by computer simulation (see the text). Note that in the case of TK_2 these permissible combinations give the reactions which are well known in cells. Abbreviations are: TKC, complexes of TK_1 and TK_2 with their one- or two-carbon unit, respectively; All, allose; Ara, arabinose; Ery, erythrose; Fru, fructose; Gla, glyceraldehyde; Glc, glucose; Rib, ribose; Sed, sedoheptulose; Xul, xylulose.

enzyme would be able to catalyze all six of these reactions. Table 2 includes all possible cases.

Let us consider now the number of carbons: a similar reasoning can be carried out reaching the same conclusion: donors of 5 and 6 carbons are involved in Reaction 1, and of 4 and 6 carbons in Reaction 2. These reactions have acceptors of 5 and 4 carbons, and 5 and 3 carbons, respectively. Therefore, any donor of 4, 5 or 6 carbons, and any acceptor with 3, 4 or 5 carbons can be a substrate for this enzyme. This capacity is schematically represented in Fig. 10(a); any combination of two of those half-reactions gives a reaction that TK_1 can catalyze. In this particular case, however, some of these must be discarded by thermodynamic reasons. In effect, the enzyme being in a metabolic system where a net flux of pentose to hexose exists, not all combinations are physically possible, since each half-reaction must occur in a given direction. [For example, in Reactions 2 and 4 of Table 2 erythrose is the donor, and the enzyme takes the C_1 unit from this sugar which becomes glyceraldehyde; but in Reaction 5 glyceraldehyde takes a C_1 unit from the enzyme complex to become erythrose; so, it is clear that Reactions 2 and 4 are incompatible with 5. Those directions which occur are also shown in Fig. 10(a). They can be easily derived theoretically by the same reasoning. Moreover, simulation of the behavior of the system by computer (see below) gave the velocities of these reactions as positive in the directions shown. Thus, the possible combinations among the thermodynamically allowed half reactions lead us to discard Reactions 5 and 6 in Table 2. TK_1 can thus catalyze Reactions 1–4.

Up to now it has been proven that the enzyme TK_1 is *able* to catalyze the first four reactions of Table 2, it being not possible to have a smaller range of specificity. Now it can also be demonstrated that if this enzyme is catalyzing Reaction 1 in a system where there is a net flux of conversion of pentose into hexose it *will* catalyze all four reactions. In accordance with the principle of activity, the enzyme will catalyze these reactions whenever their substrates are available. Since we have the substrates of the first one (ribose and arabinose), Reactions 1 and 3 occur, and they give erythrose which promotes Reactions 2 and 4.

Thus, the reasoning presented demonstrates that the pathway shown in Figs 6(b) and 8 cannot occur, since an enzyme capable of catalyzing *only* Reactions 1 and 2 in Table 2 cannot exist. That is, if Reactions 1 and 2 exist then also Reactions 3 and 4 exist. These conditions are not met in the schemes of Fig. 6(b) and 8, so these schemes are not possible.

9. Does Cellular TK_2 Fulfill These Same Principles?

Let us see now if the same reasoning when applied to cellular TK_2 and transaldolase (TA) leads to the known metabolic pathway of the present cells shown in Fig. 7. Cellular transketolase (TK_2) catalyzes the two reactions shown there. The specificity of this enzyme is stated by the following features (see Table 1): the donor of the C2-unit is a ketose with its third carbon in S configuration, and the acceptor is an aldose without any particular chirality condition. By applying the same reasoning as for TK_1 it can be proven that: (i) in a system with a net flux of conversion of pentoses into hexoses, only the two familiar reactions catalyzed by TK_2 can occur; (ii) it is not possible to reduce the range of specificity of TK_2; this enzyme catalyzes two different reactions in metabolism, not because it has a poor specificity, but because it is not possible to have more.

In effect, by applying the same reasoning as was used for TK_1 we obtain the three reactions shown in Table 3, as all the possible combinations among the two half-reactions shown in Fig. 10(b); the third eaction (fructose + ribose ↔ erythrose + sedohep-tulose) is discarded for the same thermodynamic considerations made above, and the two remaining reactions are precisely those that work in the cellular pentose phosphate pathway (Figs 1 and 7). A similar reasoning can be done for transaldolase reaching the same conclusion: the reaction of this enzyme is the only one possible. In cellular metabolism transaldolase catalyzes one reaction and transketolase two, but

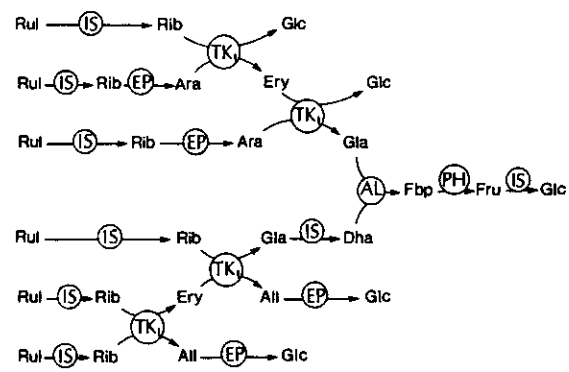


FIG. 11. Scheme which shows the complete stoichiometry of the non-oxidative phase of the pentose phosphate cycle as would occur if TK_1 were been selected. While the mechanisms shown in Figs 6(b) or (c) and 8 are not physically possible, this one is, since it includes all four reactions that TK_1 catalyzes (they are numbered as in Table 2). This mechanism has 18 steps, the same as that shown in Fig. 8, but in contrast to Fig. 8, this mechanism is physically possible. However, note that this scheme is not symmetrical (the two halfs have different reactions). So, although it has less steps than the cellular one (Fig. 7), and might look simpler, it has more different reactions. The cell scheme, selected in the evolution, is symmetrical and is more kinetically efficient.

TABLE 3
Set of all possible reactions which can be catalyzed by the cellular transketolase and transaldolase

Transketolase (TK_2)	
(1)	Xylulose + Ribose ↔ Glyceraldehyde + Sedoheptulose
(2)	Xylulose + Erythrose ↔ Glyceraldehyde + Fructose
(3)	Fructose + Ribose ↔ Erythrose + Sedoheptulose
Transaldolase (TA)	
[1]	Sedoheptulose + Glyceraldehyde ↔ Erythrose + Fructose

in these two cases, the range of specificity of these enzymes is as reduced as physically possible. So this reasoning also proves that evolution has achieved the most reduced range of specificity possible for these two enzymes. Transketolase is one of the rare cases in metabolism of the same enzyme catalyzing two different reactions (see Figs 1 and 7). Now, it is clear that it is not an enzyme with poor specificity. It catalyzes two different reactions since the mechanism of its reaction cannot have a more restricted specificity. Nevertheless, note that this situation does not establish any problem for optimizing the design of the pentose phosphate cycle.

10. The TK_1 Pathway

The simplest possible pathway for a whole conversion of pentoses into hexoses, involving the four reactions of TK_1 is that represented in Fig. 11 (irreducible, since each TK_1 reaction occurs one time). Many other combinations among these reactions can be made in order to build a solution but this is the simplest solution possible. This scheme was constructed according to the kinetic behavior of the system simulated by computer (see below for details). We must emphasize that no particular constraint on stoichiometry was imposed upon TK_1 or TK_2 systems in developing their respective stoichiometric structures shown in Figs 7 and 11. Programs were written with differential equations describing the particular enzyme reactions of Fig. 10. Then, the systems being at steady state, the stoichiometric structure of Fig. 11 was derived for the TK_1 system (using the velocity values of each individual reaction). This demonstrated a good agreement with theoretical predictions. Reactions of Table 2, discarded by thermodynamic reasons, were verified not to operate. Equivalent results were obtained by simulation of TK_2 system; it produced the stoichiometry of Fig. 7, and Reaction 3 in Table 3, discarded by thermodynamic reasons, was shown not to operate. So the pathway of Fig. 11 (*not* that of Fig. 8) is the solution physically possible which has to be compared with the cellular solution of Fig. 7. Comparing these two mechanisms we can see that the TK_1 has 18 steps, 11 different reactions and 10 different intermediates, while the TK_2 has 20 steps, nine different reactions and ten different intermediates. So, in a certain way, the TK_1 system looks simpler since it has less steps. However, it has more different reactions. Thus, the question is again: which of these two systems is simpler, and what is the reason why the TK_2 has been selected by evolution rather than TK_1? Is this in disagreement with the first part of this work which says that the shortest pathway should be selected?

11. Computer Simulation Experiments

As a result of the above ambiguity, the kinetic behavior of the two systems was simulated to determine their catalytic efficiency, i.e. which of them could yield more flux in the whole conversion of pentoses into hexoses. This was done in two experiments (one for each system) of computer simulations, where an initial substrate X_0 was converted into ribulose, and this into glucose through the appropriate set of reactions involving TK_1 or TK_2, respectively. The two systems were run under the same environmental conditions, including the same concentration of the initial substrate X_0, and the same thermodynamic and kinetic parameters. X_0 (and not ribulose) was stated as the initial substrate, and a reaction to convert X_0 into ribulose was defined; the reason for this was that ribulose has to be converted into different pentose intermediates in accordance with the system. So, conversion of X_0 into ribulose was very useful in obtaining an appropriate value of the net input flux. Output flux was defined as a first-order reaction in glucose concentration. All reactions (even the input flux) were reversible, with the exception of the last one (output flux). In this way glucose was irreversibly converted into a last sink product. Concentration of X_0 was stated as 10 (constant value) in the two systems. Equilibrium constants were 1 for isomerases, epimerases, transketolases (TK_1 and TK_2), transaldolase and aldolase, 10 for input reaction, and 1000 for

the fructose bisphosphatase (Reich & Sel'kov, 1981). The value of all microscopic velocity constants was 1 with the exception of input and output velocities which were 10, and fructose bisphosphatase which was 1000, all of these for the forward direction. These values, although realistic, do not influence the results at all since the whole equilibrium constant is the same for the two systems. Each enzymatic reaction of pentose- and hexose–isomerases, epimerases, transketolases, and transaldolase was considered to occur in two steps: the first to form the complex and the second to release the product(s), (see Fig. 10); total amount of each enzyme was taken into consideration, in accordance with the results of the first part of this work; these values (stated in order to put the two systems within the same conditions) were as follows: ribulose 6-P isomerase and glucose 6-P isomerase, 50 each in the two systems; ribose 5-P 2-epimerase and ribulose 5-P 3-epimerase, 50 each in the TK_1 system, and 100 for the only ribulose 5-P 3-epimerase in the TK_2 system; transketolase (TK_2) and transaldolase, 50 each in the TK_2 system, and 100 for the only TK_1 in its system. Amount of triose phosphate isomerase, aldolase and fructose (bis-phosphate) phosphatase were not taken into consideration (their role was the same in the two systems). The progress of each system was monitored by comparing input and output fluxes of carbon conversion. ($V_{in} \times 5$, and $V_{out} \times 6$, since six pentoses are converted into five hexoses). Steady states were considered reached when the difference between these values was less than 0·01% in each system. The results of these experiments are shown in Table 4. The TK_2 system (cellular) was shown to have more catalytic efficiency than the TK_1, giving a flux of carbon conversion of 22% over the TK_1 system.

These results explain the reason why TK_2 was selected in evolution rather than TK_1. It is clear that the pathway which TK_2 promotes has more stoichiometric simplicity, and is catalytically more efficient. However, this is an empirical result whose theoretical explanation is not obvious since the TK_1 pathway has 3·00 steps per pentose converted, while TK_2 has 3·33. The TK_2 pathway seems to be longer. Is this any contradiction with Theorem 1? What is the theory which can explain these empirical results?

TABLE 4

Kinetic behaviour of the TK_1 and TK_2 systems (see Figs 7 and 11) for the conversion of pentoses into hexoses; see "Computer simulation experiments" for details. Results of these experiments show that TK_2 system (cellular pentose pathway) with a transketolase which transfers a two-carbon unit is more efficient than the hypothetical TK_1 system based on a transketolase which transfers a one-carbon unit.

Steady-state values of fluxes and intermediates

(a) *Fluxes of conversion*

	TK_1 system	TK_2 system
Input flux (pentoses)	38·72	47·31
Output flux (hexoses)	32·25	39·38
Flux of carbon conversion†	193·64	236·54

† Pentose flux × 5, or hexose flux × 6

(b) *Concentrations of intermediates in the TK_1 and TK_2 (cellular) systems for pentose pathway (all monophosphorylated with the exception of fructose bis-phosphate)*

	TK_1 system	TK_2 system
Ribulose	61·27	52·67
Ribose	6·92	26·94
Arabinose	3·71	—
Xilulose	—	26·95
Erythrose	7·12	14·96
Sedoheptulose	—	74·09
Allose	6·22	—
Glyceraldehyde	7·33	8·79
Dihydroxiacetone	0·88	0·90
Fructose	4·48	40·61
Fructose bis-P	0·011	0·048
Glucose	3·22	3·94
Total	101·16	249·90

12. Theoretical Explanation

These results can be theoretically explained as follows. The property presented above (Theorem 1), that the short pathway yields more flux, has been shown to be a consequence of the concentration of intermediates descending along the pathway at steady state. So, the last intermediate has a smaller concentration in the long pathway than in the short one. As a consequence of this, the velocity of the last enzyme (and so the net flux) is less in the long pathway (see the derivation of Theorem 1). In the case that we consider now, the flux difference between these pathways is a consequence of the same effect. In the TK_1 pathway the more complicated stoichiometry produces an effect of intermediate dilution even though the number of different intermediate species is the same. The TK_1 pathway has more different *reactions* than the TK_2. TK_1 catalyzes in its pathway four different reactions, while TK_2 only two. So, in the TK_1 system the same intermediate is used for many purposes, which means that there are many different reactions competing for the same substrate. This situation produces lower substrate concentrations and consequently a lesser degree of enzyme saturation and well explains the lower activity of the enzymes

and the decreased output flux. It is interesting to observe that the same reasoning as used above to prove Theorem 1, based on mass action, demonstrates now how stoichiometrical simplicity is catalytically more efficient. The same simulation experiments described above yielded results which demonstrate these differences within pools of the two systems. The intermediate concentrations at steady state in the two systems were checked by computer simulation. These results are shown in Table 4(b), where it can be seen that as theory predicts the concentration of sugar intermediates in the TK_2 system was greater than in TK_1.

13. The Role of Symmetry in Metabolism

There is an important difference between the TK_1 and TK_2 systems which deserves to be mentioned. The cellular (TK_2) solution is symmetrical as can be in Figs 1 and 7; this means that the same set of reactions take place in each half of the whole stoichiometry. In contrast, the solution of the TK_1 system (Fig. 11) is not symmetrical, i.e. different sets of reactions are involved in each half. Symmetrical solutions based on TK_1 could be possible, but not with all the four reactions working at the same velocity. The reason is as follows: Each half must involve $nC_5 \rightarrow mC_6 + C_3$, which can be written as $5n = 6m + 3$; $6m$ is always even, therefore $6m + 3$ is odd, and $5n$ must also be odd, which means that n is odd. But the four TK_1 reactions, each one of them occurring once, convert *six* pentoses (see Table 2). So, they cannot account for an odd number of pentoses. It can be seen that in the cell solution all the possible reactions of TK_2 and TA occur the same number of times and the system has a symmetrical structure. Such an arrangement is not possible with TK_1. The metabolic importance of symmetry is clear in light of the reasoning developed above. A symmetrical organization of metabolic pathways saves enzymes and takes better advantage of the intermediate pools, which results in a more economic and efficient machinery. Thus, its selective advantage is clear.

14. Discussion

The major emphasis of this work is the following: This study on the organization of the pentose phosphate pathway demonstrates analytically the process of optimization which occurred during the evolution of metabolism. It is clear that the target of this optimization was the maximization of flux. This was not an easy problem for cellular evolution to solve. It has been shown here that there were many different possibilities to check in order to build a kinetically efficient metabolic system for converting pentoses into hexoses. In effect, the present design of the pentose phosphate pathway has involved: (i) the choice of the most appropriate mechanism for carbon transfer (thiamin pyrophosphate), (ii) the construction of an enzyme using this mechanism (transketolase), (iii) the choice of the number of carbons to be transferred (TK_2) which guarantees the minimization of the possible reactions to be catalyzed by the enzyme, (iv) the process of reduction of enzyme specificities in order to avoid useless reactions which dissipate mass efficiency, and (v) the construction of other "auxiliary" enzymes (isomerases, epimerases, etc) for supplying the appropriate pentose substrates for carbon transfer mechanisms. Every one of these stages in the evolutionary process had to be based upon a previous one. This again shows the tortuous and difficult path for optimizing variables. These variables cannot be improved upon. Indeed, natural selection is a very good mechanism for optimization.

The reason why simplicity has been a target in evolution is clear with these results: simplicity implies more catalytic efficiency. Also these results demonstrate the close relationship between simplicity and economy. Our results give a good explanation of why the enzymes for pentose metabolism are precisely those seen in modern cells. In previous papers on this subject (Meléndez-Hevia & Isidoro, 1985; Meléndez-Hevia & Torres, 1988; Meléndez-Hevia, 1990), simplicity and mechanisms were considered as hypotheses, without any particular justification. Now, after this research all previous hypotheses are no longer arbitrarily imposed, but are *results*. It is clear, and it must be emphasized, that if we wished to construct a metabolic pathway to convert pentoses into hexoses obtaining the greatest possible flux and enzyme economy, and if we had absolute liberty to choose any feasible chemical mechanism and to state the enzyme specificity, with no more constraint than elementary physical and chemical principles, we would not be able to find any better route than the modern pentose phosphate cycle.

It is well known that Darwin's theory has been frequently criticised by arguing that it contains circular reasoning; i.e. Natural Selection leads to "survival of the fittest", but a problem appears when trying to answer the question "who are the fittest?" because in most cases one does not have any criterion other than to say "those that survive". We think that Darwin's theory does not necessarily lead to circular reasoning since the problem is only in the definition and quantification of fitness. Fitness is a broad concept, easily used in an ambiguous manner. Even so, there

are many cases where fitness can be well defined, but not numerically measured. We would like to emphasize that in this work fitness is clearly defined as a set of variables numerically measurable, namely, the least number of enzyme reactions, with a clear physiological meaning of catalytic efficiency. Therefore, there is no ambiguity in this case.

We have considered here that the maximization of flux was the target of the optimization process. Obviously, there can exist a number of other goals for an organism. It is clear that since different population groups may also have different problems to resolve, and consequently different variables to be optimized, evolution can lead to different solutions, and is necessarily divergent. This fact in no way contradicts the reasoning developed herein.

Also it is clear that until the modern, optimized metabolic design was achieved, many organism-types had to function under other less-optimized designs, which can formally be called *paleometabolism*. Most parts of paleometabolism have been lost resulting in the familiar general map of metabolic pathways present in modern species. However, it is well known in paleontology that a number of now living species have existed from about 50–400 million years ago. These paleospecies (also, although improperly, called "living fossils"), survivors of massive extinctions, are living representatives of groups less evolved, see, for example, (Eldredge & Stanley, 1984). Perhaps pathways of paleometabolism might be found in them, in the same way that they keep a number of morphologic features characteristic of less evolved stages. It must also be taken into consideration that a number of targets in the optimization process could have been particular and accidental, simply in accordance with requirements of some groups under special environmental conditions. This could promote a certain tendency of metabolic evolution to optimize a different variable in some restricted group, and could have promoted their extinction—bad chemical machinery when the old target was no longer useful. Also it must be considered that in the long and tortuous way for optimizing metabolism, a number of non-optimized metabolic designs could have been developed, some of them with provisional selective advantages, such as a channeling built on a non-optimized pathway (Meléndez-Hevia & Montero, 1991); it is catalytically more effective than the same design without channeling, but is, in fact, a local basin whose design (reaction sequence) has no possibility to be improved. Thus, it could explain the (temporary) success that some groups had as well as their further extinction.

It is reasonable to think that many of the most important (and primitive) problems in metabolic design were solved in the earliest stages of the evolutionary process. Nevertheless, some metabolic problems can have appeared much later, since evolution has also produced new necessitites to be met by the chemical machinery [such as the metabolic adaptation to very dry climates in Crassulaceae, which involves a new special pathway (Ting, 1985)]. Thus, some remainders of paleometabolism could be found. In fact, the presence of the transaldolase in chloroplasts is a good example of paleometabolism. The Calvin cycle was derived from the pentose phosphate cycle, and the adaptation of this pathway for the new purpose of conversion of 12 trioses into six pentoses plus one hexose has involved the elimination of transaldolase, as a condition for optimizing the design. Inclusion of this enzyme in the Calvin cycle would have obliged the carbon flux to flow through a longer route, as it has been proven by Meléndez-Hevia (1990). Transaldolase has been found in chloroplasts of spinach and peas (Latzko & Gibbs, 1968; Anderson, 1981) but its role in the Calvin cycle is small because *it is inactivated by light* (Anderson, 1981). This is, therefore a clear case of paleometabolism. The present (modern) Calvin cycle is a well-optimized pathway, but some earlier stages of its optimization process have been able to be found.

This paper explains what may be interpreted as paleometabolism in the pentose phosphate cycle. It is clear that an equivalent theory on the optimization of other metabolic pathways will be necessary in order to identify paleometabolism elsewhere. We believe that the discovery of more cases of paleometabolism could be possible once one has a suitable theory to guide the search.

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