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Irreversible Metabolic Transitions: The Glucose 6-Phosphate Metabolism in Yeast Cell-Free Extracts

Marie-Agnès Coevoet and Jean-François Hervagault[1]
UPRESA n° 6022 du Centre National de la Recherche Scientifique, Université de Compiègne, B.P. 20529, 60205 Compiègne, France

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The steady-state and dynamic behavior of a partial glycolytic reaction sequence are investigated in cell-free extracts of yeast. Pyruvate kinase, adenylate kinase and glucose 6-phosphate isomerase cooperate to a multienzyme system centered around the 6-phosphofructokinase (6-PFK) and fructose 1,6-bisphosphatase (FBPase) cycle. The reaction system operates under thermodynamically open conditions maintained by a continuous supply of substrates, i.e., glucose 6-phosphate (Glc6*P*), ATP and phospho*enol*pyruvate (*P*Prv) in a flow-through reaction chamber. Appropriate conditions lead to the occurence of (two) coexisting and markedly different time-independent states in the metabolite concentrations and fluxes. For particular experimental conditions, changes in the influx adenylic energy charge, $[AEC]_{IN}$, may cause transitions between these alternative steady states which are either reversible as it occurs in classical hysteresis phenomena, or, more importantly, irreversible (irreversible transitions, IT) where the system is not able to switch back to its previous state even when the perturbation is reverted. The emergence of these irreversible transitions do not result from artificial or non-realistic experimental constraints, but are a potential intrinsic property of any non-linear dynamic system exhibiting bi- or multistability. These one-way transitions may well have important biological implications with respect to switching, adaptation and memory phenomena. © 1997 Academic Press

Substrate cycles are ubiquitously distributed throughout metabolic pathways. They take place in many instances such as equivalent reduction and oxidation, synthesis and degradation and in the generation of energy. The covalent modification of proteins by interconverting enzymes such as kinases and phosphatases is also an important feature of cycles involved in signalling pathways. The roles played by cycles in the regulation of metabolism is still a matter of controversy. However, from experimental data and theoretical considerations, it is likely that substrate cycling is physiologically involved in thermogenesis, in the orientation of fluxes, in the control of concentrations and in the amplification of sensitivity (1-7). When dealing with minimum cycles where a reaction intermediate either triggers its own synthesis (product activation) or inhibits its own degradation (substrate inhibition), then, more sophisticated steady-state and dynamic behaviors such as multi-stability (alternative steady states), sustained temporal oscillations or chaotic motion may be expected from theoretical considerations (8, 9). The more familiar feature related to bistability is the emergence of reversible hysteretic transitions between two functionally different branches of steady states in response to a change in a control parameter value: these abrupt transitions (infinite amplification) may occur for two different threshold values of the parameter considered (limit points, LP's). Thus, in addition to their ability in storing information (short-term memory), such bistable substrate cycles can be regarded as powerful regulatory devices with dual capacities by either supporting the maintenance of metabolite levels and fluxes (homeostasis) or amplifying sensitivity (switching effect) in response to a regulatory signal (10-12). Moreover, under realistic conditions, these (non self-releasing) triggers may loose their ability to switch reversibly from one steady state to the other. If one of the limit points either disappears or is no more physically accessible, then, a transition between the two branches of steady states is only possible one time and from that moment on, the system is unable to trace back its previous history and selects thus one mode of operation in an irreversible manner (13-15). Surprisingly, little attention was paid so far to the biological significances and implications of these irreversible transitions as well as their experimental search. A model of isothermal chemical reactions (Michaelis-

[1] To whom correspondence should be addressed. Fax: (33) 03 44 20 39 10; e-mail: jean-francois.hervagault@utc.fr.

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Menten mechanism) occuring in a volume bounded by a membrane and immersed in a reservoir of substrates and products at fixed concentrations has been theoretically analyzed: if the permeability of the membrane to a given species is taken to be a non-linear function of the concentration of that species, then the coupling between reaction and diffusion provides feed-back and IT between stable branches of steady states can be induced (16-19). In the frame of a mathematical model for the glycolysis of human erythrocytes and taking into account ATP synthesis and consumption, it was shown that below a critical load of ATP consumption the system could exist under three different states, i.e. a physiological ATP-producing state, a saddle point (unstable) and a stable non-energized steady state (ATP = 0). Above a critical level of energy consumption, the system breaks down irreversibly to the non-energized state. The initial state cannot be restored by decreasing the rate of ATP consumption (20).

Glycolysis is a prototype of non-linear dynamic pathways. There is general consensus that the 6-PFK / FBPase (fructose 1,6-biphosphatase) cycle is a core element of the glucose metabolism and the main source of non-linearities through allosteric regulation by AMP and fructose 2,6-bis-phosphate, acting synergetically as activators and inhibitors on 6-PFK and FBPase, respectively (21, 22). Dynamic hysteretic transitions, damped and sustained oscillations of the concentrations of glycolytic intermediates were demonstrated experimentally in yeast and muscle whole cells (23-26). These various behaviors were also observed when dealing with cell-free extracts from yeast and liver and agreed both qualitatively and quantitatively with the predictions of simple theoretical models taking into account the kinetic properties of the 6-PFK and FBPase cycle when operating under open conditions (27,28).

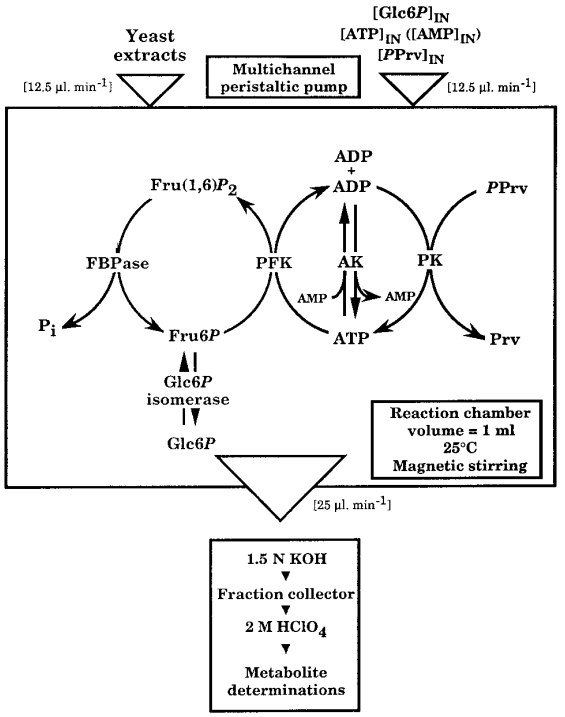

FIG. 1. From two reservoirs, the buffered cell-free extracts and substrates are continuously injected with equal flow-rates (12.5 μl min^{-1} each) into a magnetically stirred flow-through reactor (volume = 1 ml, i.e., τ = 40 min). Due to the tight linkage between the transmembranal transport and phosphorylation of sugars in yeast, glucose 6-phosphate is added as the substrate instead of glucose. A pool size of 3 mM is maintained for the adenine nucleotides. The reactions are driven by the regeneration of ATP from externally added phospho*enol*pyruvate, *P*Prv. The functional state of the system is reflected by the actual metabolite concentrations within the reactor and determined in the efflux solution (flow-rate = 25 μl min^{-1}) after immediate inactivation of enzymatic processes by 1.5 N KOH with further neutralization by 2N $HClO_4$. All experiments are carried out at 25°C in a 100 mM imidazole/HCl buffer, pH 6.6, containing 100 mM KCl, 8 mM $MgCl_2$, 10 mM K_2HPO_4, 50 μM EGTA and 2.5 μM mercaptoethanol.

MATERIALS AND METHODS

Cell-free extracts preparation. The yeast strain *S. cerevisiae* DFY1 (D585-11c) is grown at pH 6.5 in a medium containing 2% tryptone and 2% glucose (29). Flasks of 500 ml containing 150 ml medium are incubated at 30°C. Cells are harvested at the end of the exponential growth phase (20 hours, A_{580} = 6 and wet weight = 8 mg ml^{-1}). After washing with cold distilled water, cells are stored frozen at −20°C. Cell-free extracts are freshly prepared on the day of the experiment. After thawing, the cells are washed three times in water and subsequently suspended in an equal volume of 20 mM K_2HPO_4, pH 6.6, containing 10 mM $MgCl_2$, 2 mM mercaptoethanol, 1 mM phenyl-methylsulfonylfluoride [PMSF, a protease and ATPase inhibitor, (30)] and 4 mM benzamidine (buffer A). Glass beads (425-600 μm diameter) are added and the suspension is vibrated for six 30-s periods with intermediate cooling at 4°C in a vortex mixer. Supernatants are then centrifuged at 100 000 × g for 45 min. The crude cell-free extracts are passed over a Sephadex G25 M column equilibrated with buffer A. The protein concentration in the eluate ranges between 3 and 5 mg ml^{-1}.

Numerical. The numerical continuation of steady-state solutions and the related bifurcation diagrams were computed by using the software package AUTO (31). The time evolution of the metabolite concentrations was calculated with a semi-implicit Gear algorithm (32).

RESULTS

When using yeast extracts, the model accounts for a reaction network operating under thermodynamically open conditions in a flow-through reaction chamber with a constant supply of extracts and substrates, i.e. hexose monophosphates (Glc6*P* + Fru6*P*), adenine nucleotides (ATP + ADP + AMP) and *P*Prv. ATP is regenerated from *P*Prv by pyruvate kinase (PK). Quasi-equilibria of the adenine nucleotides and hexose monophosphates in the time scale of the experiments are maintained by the presence of high concentrations of adenylate kinase (AK) and Glc6*P* isomerase (Fig. 1). Therefore, the global system can be modeled by a set of three coupled differential equations for the metabolite

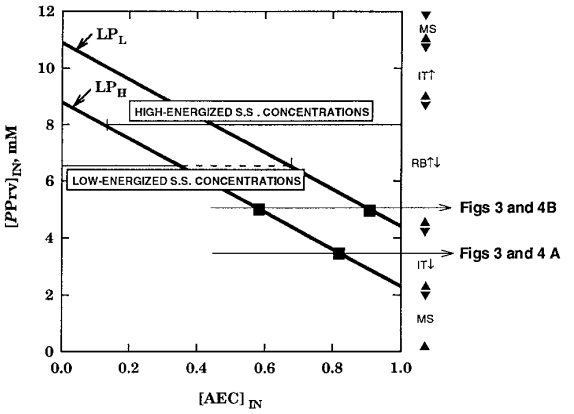

FIG. 2. Locus of limit points in the $[AEC]_{IN}$ / $[PPrv]_{IN}$ parametric plane. On the right-hand side of the figure are indicated the various behaviors observable for different domains in the phospho*enol*pyruvate influx concentrations (MS: monostability, zero LP; RB ↑↓: reversible bistability, two LP's; IT↑ and IT↓: irreversible up- or downwards transitions at LP_L and LPH_H, respectively). [The full squares shown on the limit point loci refer to Fig. 3]. When varying the influx adenylic energy charge (as well as the influx *P*Prv concentration), the branch of solutions corresponding to the high energized steady-state concentrations (high ATP and *P*Prv) always overlap the alternative branch (this overlapping domain is symbolized by a dashed segment). Parameter values are as follows: $[Glc6P]_{IN}$ = 7.5 mM, $[ATP]_{IN}$ = 3 mM, τ = 40 min, v_{PFK} = 0.7 U ml^{-1}, v_{PBPase} = 0.015 U ml^{-1}, v_{PK} = 10 U ml^{-1}, v_{AK} = 4 U ml^{-1} and v_{PGI} = 3 U ml^{-1}.

pool concentrations ([Fru6*P*] + [Glc6*P*], {2[ATP] + [ADP]{ and [*P*Prv]), taking into account the actual enzyme kinetics and flow processes and complemented by a set of algebraic relations reflecting the conservation of the metabolite pools and quasi-equilibria equations (28, 33, 34).

Thorough numerical investigations of that model led us to predict that irreversible transitions might be observed when the influx adenylic energy charge, $[AEC]_{IN}$, is taken as the control parameter (35). Therefore, the continuation of limit points (Fig. 2) in the energy charge of the adenylate influx *vs* the phospho*enol*pyruvate influx concentration parametric plane ($[AEC]_{IN}$ / $[PPrv]_{IN}$) shows that zero, one or two limit points (LP's) are crossed by a representative point of the system when the $[AEC]_{IN}$ is varied between zero and unity (bounded domain, by definition). The advancement of the metabolite concentrations will thus be either monotonously continuous (zero LP, monostability), or discontinuous (one or two LP's) under the form of sudden jumps when passing a LP. The norms of the concentration vector, η, plotted as a function of the $[AEC]_{IN}$ for two characteristic $[PPrv]_{IN}$ values (Fig. 3), illustrate on the one hand a classical hysteretic bistability ($[PPrv]_{IN}$ = 5 mM, two LP's) and, on the other hand, a case of irreversible transitions ($[PPrv]_{IN}$ = 3.5 mM, one LP). In this latter situation, the abscissa of LP_L is greater than unity and is thus not accessible experimentally, i.e. this limit point has to be regarded as a virtual entity in that it occurs in a non-physical range of the parameter.

From these numerical predictions, and given an initial influx energy charge close or equal to unity, the experimental confirmation for the existence of these irreversible transitions may consist in starting up the experiment under conditions where the metabolite concentrations are high, that is, close to the influx concentrations. The energy charge is then decreased down to a value below the LP_H abscissa. Once the new steady-state regime is reached, the influx energy charge is increased up again to unity. If the three metabolite pool (steady-state) concentrations return to their initial value, then the behavior of the system is of hysteretic nature (Figure 4B), or, if they establish to different values, then the perturbation in the influx energy charge has provoked an irreversible transition in the metabolite concentrations and further changes in the $[AEC]_{IN}$ will maintain the system infinitely in a low-energized state. The experimental evidence for the occurence of these irreversible transitions is illustrated and detailed in Figure 4A.

DISCUSSION AND CONCLUSION

The resolute choice of such a minimal system in which all kinetic and regulatory properties are well-defined allows (i) the derivation of a realistic and robust model with predictive abilities and (ii) an experimental investigation under strictly controllable conditions. The results obtained with such an over simplified experimental model address naturally the issue of their relevance facing the complexity of metabolism and what may actually occur within the living cell: (i) our

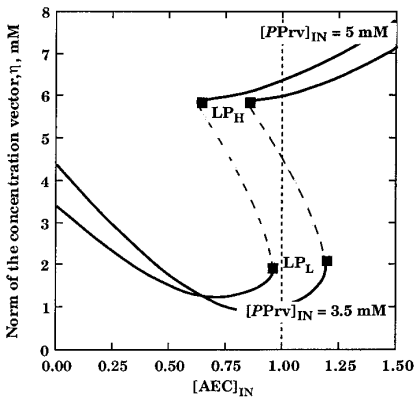

FIG. 3. Norm of the steady-state concentration vector η, plotted as a function of the influx energy charge, $[AEC]_{IN}$ for two characteristic $[PPrv]_{IN}$ concentrations, i.e., 3.5 and 5 mM. All other parameters have the same value as in Fig. 2. The portions of curves bounded by the two limit points, LP_H and LP_L refer to unstable solutions (saddle points) and are not, therefore, reachable experimentally under normal conditions. Similarly, any point with abscissa greater than unity has no physical meaning.

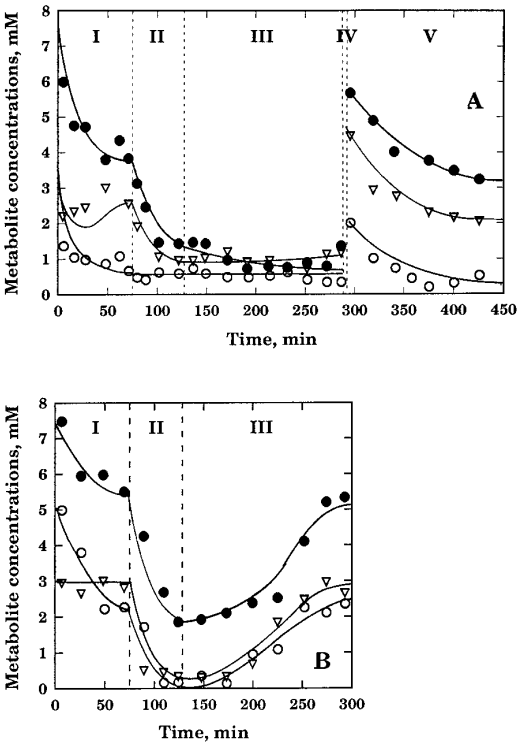

FIG. 4. **(A)** Time evolution of the metabolic pool concentrations as experimentally observed and demonstrating the occurence of irreversible transitions in yeast cell-free extracts ($[PPrv]_{IN}$ = 3.5 mM). Symbols ●, ▽ and ○ refer to the actual hexose monophosphate (Glc6*P*+Fru6*P*), ATP and *P*Prv concentrations within the reactor, respectively. The parameter values are the same as those used in the numerical simulations shown in Figures 2 and 3. A stable steady state is approached at the end of phase I. This state is characterized by high hexose monophosphate (HMP) and ATP concentrations. The latter is correlated with low concentrations of ADP and AMP, and hence with a high energy charge (close to 1). This state is therefore denoted as a high-energized steady state. In phase II, the system is perturbed for 50 min by supplying the adenine nucleotides under the form of AMP ($[ATP]_{IN}$ = 0, $[AMP]_{IN}$ = 3 mM). In accordance with the bifurcation diagrams shown in Fig. 3, a limit point, LP_H, is passed by and the concentrations of HMP and ATP decrease continuously down to very low values, defining a new stable and low-energized steady state. Then, in phase III, in the influx, AMP is replaced again by ATP (experimental conditions as in phase I). Although the $[AEC]_{IN}$ has returned to its upper possible value ($[AEC]_{IN}$ = 1), a low energized stationary state is approached again. This proves the irreversibility of the transition to low energized stationary states caused by a transient decrease of the influx adenylate energy charge. Any attempt to switch back to the coexisting high-energized stationary state would require a perturbation of the metabolic system by other experimental parameters: in phase IV (2 min), an overload in the influx *P*Prv, ATP and Glc6*P* concentrations to 50, 30 and 75 mM, respectively, results in an increase of the three metabolites. Then, after restoring the original experimental conditions, the original high-energy steady state is approached. The metabolite concentrations resemble those of phase I. Indeed, as shown in Fig. 3, an increase in the $[PPrv]_{IN}$ results in a decrease of the LP_L abscissa, i.e., from $[AEC]_{IN} > 1$ to $[AEC]_{IN} < 1$ (experimentally meaningful) and the diagram is representative of a classical reversible bistable (dynamic hysteresis) as experimentally shown in Fig. 4 (B)**(B)** Same experiment as above (phases I to III) but for a slightly higher $[PPrv]_{IN}$ value (5 mM), all other parameters being unchanged. Here again, phases I and III refer to an influx of ATP 3 mM, whereas phase II refers to an influx of AMP 3 mM. Contrarily to what occured in the previous experiment, the replacement of AMP by ATP (II → III) drives the whole system back to its initial situation (end of phase I).

extracts are free from low molecular weight compounds to preclude bypass reactions to the investigated glycolytic segment and covalent modifications of the enzymes. In particular, the presence of Fru(2,6)P_2, the most potent allosteric effector of PFK and FBPase, was excluded. Therefore, further studies of the Fru6*P*/ Fru(1,6)P_2 substrate cycle should include extracts containing the 6-phosphofructo-2-kinase/ fructose 2,6-bisphosphatase. By this way, the dynamic complexity of the system would be considerably enhanced (36, 37), (ii) in our model system, the *P*Prv and adenylate fluxes are taken as external parameters whereas they might be seen as *in vivo* internal variables.

If a straightforward extrapolation from regulatory pattern observed in cell-free extracts to the actual metabolism has to be handled with caution, it remains true, however, that some of the observed qualitative features resemble physiological situations encountered within the cellular metabolism. In particular, the interpretation of numerous experimental works published some decades ago and reporting observations of *irreversible metabolic transitions* in microorganisms might probably be revisited in the frame of the multistable dynamic systems (38-41).

It was recently shown theoretically, that the model network studied in this report might also exhibit so-called *non-connected branches of alternative steady states*, i.e. situations where any transition between the two stable branches of the bistable are physically impossible (42). Such structures produce functionally equivalent or reciprocal (stable) states with distinct metabolite pattern and hence may either serve as a prerequisite for distinct evolutionary paths or define events for distinct transformation of cells. The existence of irreversible transitions in metabolic structures exhibiting bistability have not to be regarded as exotic phenomena but rather as either powerful devices used by the cell for the selection and/or adaptation of pathways, or consequences of disregulations in the normal operation of the cellular metabolism (mutations, pathologies, . . .). As an illustration, a putative mechanism was proposed for a molecular bistable switch that can store information indefinitely, despite the complete turnover of the molecules that make up the switch (43). This model might apply, for instance, to the interconversion of specific proteins such as synapsin or tau by the Ca^{++}/ calmodulin-dependent protein kinase II (CaMKinaseII) / Calcineurin couple and account at least in part for the occurence of long-term potentiation and/or depression (LTP/LTD) in synapses.

In 1949, Max Delbrück stated (44) that *biological systems under flow equilibrium conditions, might be*

capable of existing in different steady states, under identical conditions. Such systems could switch from one steady state to another under the influence of transitory modifications of the environment ob. . .]. These transitions might be either reversible or irreversible as it occurs in the differentiation processes.

REFERENCES

1. Atkinson, D. E. (1977) Cellular Energy Metabolism and Its Regulation, Academic Press, New York.
2. Newsholme, E. A., and Crabtree, B. (1976) *Biochem. Soc. Symp.* **41,** 61–109.
3. Hofmeyr, J. H. S., Kacser, H., and Van der Merwe, K. J. (1986) *Eur. J. Biochem.* **155,** 631–641.
4. Newsholme, E. A., and Start, C. (1973) Regulation of Metabolism, John Wiley, London.
5. Stadtman, E. R., and Chock, P. B. (1978) *Curr. Top. Cell. Regul.* **13,** 53–95.
6. LaPorte, D. C., and Koshland, J., D. E. (1983) *Nature* **305,** 286–290.
7. Cimino, A., and Hervagault, J. F. (1987) *Biochim. Biophys. Res. Comm.* **149,** 615–620.
8. Reich, J., and Sel'kov, E. E. (1981) Energy Metabolism of the Cell: A Theoretical Treatise, Academic Press, New York.
9. Ricard, J., and Soulié, J. M. (1982) *J. Theor. Biol.* **95,** 105–121.
10. Goldbeter, A., and Koshland, Jr., D. E. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 6840–6844.
11. Goldbeter, A., and Koshland, Jr., D. E. (1982) *Quart. Rev. Biophys.* **15,** 555–591.
12. Okamoto, M., Sakai, T., and Hayashi, K. (1988) *Biotechnol. Bioeng.* **32,** 527–537.
13. Hervagault, J. F., and Canu, S. (1987) *J. Theor. Biol.* **127,** 439–449.
14. Hervagault, J. F., and Cimino, A. (1989) *J. Theor. Biol.* **140,** 399–416.
15. Fassy, F., Mandon, M., and Hervagault, J. F. (1992) *Biosystems* **26,** 159–169.
16. Blumenthal, R., Changeux, J. P., and Lefever, R. (1970) *J. Membr. Biol.* **2,** 351–359.
17. Babloyantz, A., and Sanglier, M. (1972) *FEBS Lett.* **23,** 364–366.
18. Hahn, H. S., Ortoleva, P., and Ross, J. (1973) *J. Theor. Biol.* **41,** 503–521.
19. Hervagault, J. F., Ortoleva, P., and Ross, J. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 10797–10800.
20. Rapoport, T. A., and Heinrich, R. (1975) *Biosystems* **7,** 120–129.
21. Hofmann, E., and Kopperschläger, G. (1982) *Methods Enzymol.* **90,** 49–60.
22. Van Schaftingen, E. (1987) *Adv. Enzymol.* **59,** 315–395.
23. Thornheim, K. (1978) *J. Theor. Biol.* **79,** 491–541.
24. Goldbeter, A., and Caplan, S. R. (1976) *Annu. Rev. Biophys. Bioeng.* **5,** 449–476.
25. Hess, B. (1983) *Hoppe-Seyler's Z. Physiol. Chem.* **364,** 1–20.
26. Hess, B., and Markus, M. (1985) *Ber. Bunsenges. Phys. Chem.* **89,** 642–651.
27. Frenzel, J., Schellenberger, W., and Eschrich, K. (1995) *Biol. Chem. Hoppe- Seyler* **376,** 17–24.
28. Eschrich, K., Schellenberger, W., and Hofmann, E. (1990) *Eur. J. Biochem.* **188,** 697–703.
29. Clifton, D., Weinstock, S. B., and Fraenkel, D. G. (1978) *Genetics* **88,** 1–11.
30. Jusic, M., Hinze, H., and Holzer, H. (1976) *Hoppe-Seyler's Z. Physiol. Chem.* **357,** 735–740.
31. Doedel, E. (1981) *in* Tenth Manitoba Conf. on Numerical Mathematics and Computation, pp. 265–284, Univ. of Manitoba, Winnipeg.
32. Gear, C. W. (1971) Numerical Initial Value Problems in Ordinary Differential Equations, Prentice Hall, Englewood Cliffs, NJ.
33. Hofmann, E., Eschrich, K., and Schellenberger, W. (1985) *Adv. Enzyme Regul.* **23,** 331–362.
34. François, J., Eraso, P., and Gancedo, C. (1987) *Eur. J. Biochem.* **164,** 369–373.
35. Schellenberger, W., and Hervagault, J. F. (1991) *Eur. J. Biochem.* **195,** 109–113.
36. Frenzel, J., Schellenberger, W., Eschrich, K., and Hofmann, E. (1990) *Biol. Chem. Hoppe-Seyler* **371,** 841–850.
37. Federov, S., and Uyeda, K. (1992) *J. Biol. Chem.* **267,** 20826–20830.
38. Rashevsky, N. (1960) Mathematical Biophysics, Dover, New York.
39. Novick, A., and Wiener, M. (1957) *Proc. Natl. Acad. Sci. USA* **43,** 553–566.
40. Cohn, M., and Horibata, K. (1959) *J. Bacteriol.* **78,** 601–612.
41. Eisen, H., Brachet, P., Pereia da Silva, L., and Jacob, F. (1970) *Proc. Natl. Acad. Sci. USA* **66,** 855–859.
42. Hervagault, J. F., and Schellenberger, W. (1993) *J. Biol. Sys.* **1,** 375–387.
43. Lisman, J. E. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 3055–3057.
44. Delbrück, M. (1949) *Coll. Int. CNRS* **7,** 25–26.