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EVOLUTIONARY MOLECULAR ENGINEERING BASED ON RNA REPLICATION

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Abstract - The self-replication of suitable single-stranded RNA molecules by the enzyme Qβ-replicase offers the possibility of generating optimized molecular phenotypes by controlled molecular evolution. In order to develop an experimental procedure for testing the potential of such evolutionary molecular engineering it is necessary to understand the reaction kinetics of the replication process and of competition among different self-replicating RNAs. Here the essential features of the kinetics are described in terms of replication models that can be tested by computer simulations of more extensive models and compared with laboratory data. The basic features of an evolution reactor capable of exploiting the properties of this self-replicative system are presented.

INTRODUCTION

The study of molecular evolution *in vitro* requires a self- replication system that is chemically simple enough to be understood and stable enough to allow experimentation under readily controlled conditions. The most suitable laboratory self-replication system found so far utilizes the replication of single-stranded RNA by the replication enzyme of the coliphage Qβ. (1) This four-subunit enzyme, only one subunit of which is actually coded for by the Qβ virus itself, can be prepared in adequate amounts in very pure form, is relatively stable, and proves to accept quite a number of RNA templates other than the viral RNA itself. Some of these, investigated in detail by Spiegelman and coworkers, are derived from the viral RNA by replication under artificial selection pressure. (2,3) Others are generated by the enzyme itself in absence of endogenous template by *de novo* synthesis and evolutionary optimization. (4,5) The mechanism of the replication process has been established by the traditional methods of molecular biology (1) and subjected to a detailed chemical kinetics analysis. (6,7) In this paper we summarize the results of the kinetics analysis and use them to describe a possible application of this self-replicative system for a new type of evolutionary biomolecular engineering.

MECHANISM OF RNA SELF-REPLICATION

The process of self-replication catalysed by Qβ replicase is shown in Fig. 1. For *in vivo* replication, the enzyme and the two complementary RNAs are of course involved in other processes as well, optimized to insure the infectiousness of the Qβ virus. For *in vitro* experiments, however, the amount of enzyme can be controlled, and the RNA concentrations then grow with time in a manner that depends only on the rate constants of the important steps of the replication process. While there are very many individual elementary steps in the replication chemistry, extensive computer simulation studies confirmed that the essential kinetic behaviour can be understood by condensing the mechanism to the four steps shown for each of the complementary cycles in Fig. 2.

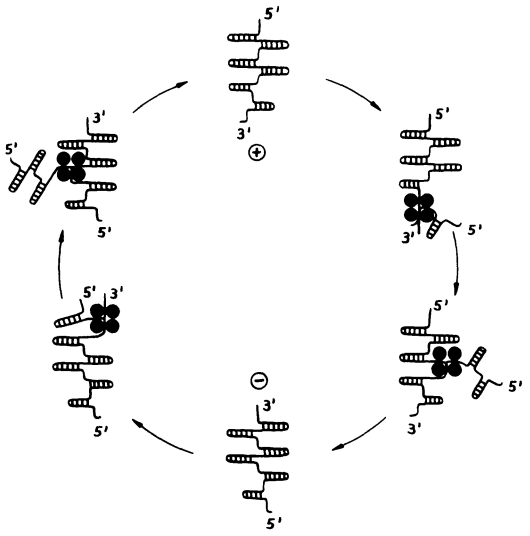

Fig. 1. Replication of single-stranded RNA by Qβ replicase. The replicase enzyme (four dots) recognizes the 3′ end of the RNA and causes the complementary replica RNA to be synthesized. The replica is released rapidly at the end of the elongation process and can then itself serve as template for a second round of replication. The slower process of template release regenerates the enzyme. (After Ref. 8)

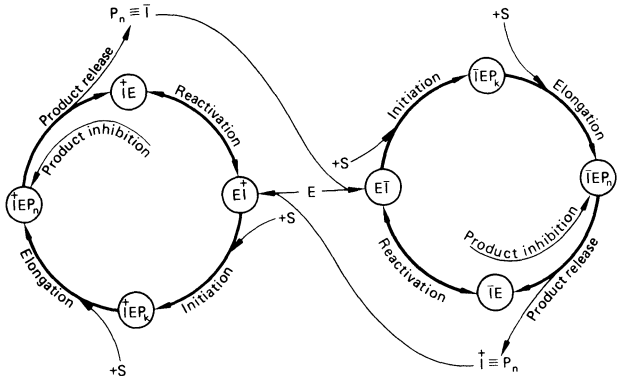

Fig. 2. Schematic reaction mechanism for self-replication of single-stranded RNA by the enzyme Qβ replicase. There are two replication cycles, one for each of the complementary strands, coupled to one another by the fact that the replica RNA synthesized in each cycle is the template for the complementary cycle. The RNA molecules are symbolized here as I (for information carrier), the replicase enzyme by E. Formation of the initial complex EI leads after formation of the first phosphodiester bond to the first of the replication complexes IEP_k. These are then elongated by further instructed addition of nucleoside triphosphates (S) to give the final replication complex IEP_n, which quickly loses the replica P_n (the I for the complementary cycle) to form the inactive complex $I\bar{E}$ in which the template molecule is bound at its 5′ end. Reactivation of the replication complex, shown here as a single step, is a slow step that has been shown to involve dissociation of the 5′-bound template and then reassociation. Also not shown here is the possible reaction of the two complementary RNAs to form double-stranded RNA that is inactive as template. (From Ref. 9)

Laboratory experiments can be conducted in various ways with Qβ replicase. The basic procedure is generally that illustrated in Fig. 3. The characteristic growth curves, measured for example by incorporation of ^{32}P-labelled nucleotides into RNA, depend in characteristic ways upon the nature of the template and the amount of template added at the start of the experiment. We shall have occasion to refer to this characteristic behaviour repeatedly.

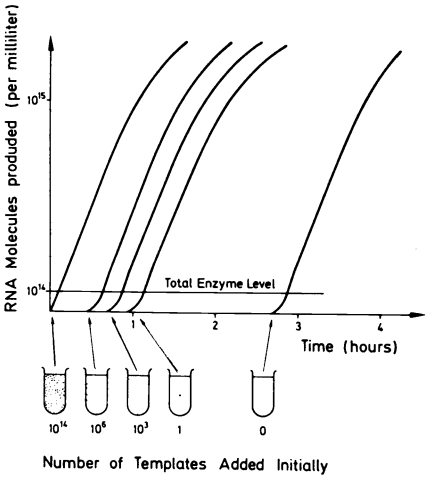

Fig. 3. Synthesis rates of RNA in typical experiments using Qβ replicase. If the incubation mixture (template RNA, Qβ replicase, nucleoside triphosphates, salt, buffer) contains concentrations of template equal to or greater than the concentration of enzyme, the synthesis rate of new RNA is constant until the RNA concentration becomes so high that product inhibition occurs. (Shown by the leveling of all curves at long times.) This can be understood as saturation of the replica site of the replicase by template. At lower template concentrations, the growth curves are displaced to longer times by amounts that depend logarithmically upon the starting concentrations of template. This reflects the autocatalytic nature of the self-replication process, i.e. the exponential growtn of template concentration that occurs when an excess of free enzyme is present. If no enzyme at all is present at the start of the experiment, the replicase is able, after a long lag time, to synthesize enough trial oligonucleotides without a template that eventually one of them has a sequence that allows it to act as an initial template that can be amplified, with concurrent evolutionary improvement, to observable concentrations. This de novo process generates a different sequence each time it occurs, but after long evolution times a single de novo product characteristic of the incubation conditions is derived. (After Ref. 10)

LABORATORY AND COMPUTER EXPERIMENTS

Insight into the interactions among the various parts of the replication process can be gained by numerical integration of the kinetic equations for a very detailed description of the reaction, including each elementary reaction separately and using rate constants consistent with all experimental information that is available. Such a computer experiment is illustrated in Fig. 4. In this semilogarithmic presentation the exponential growth character of the replication process is seen to pertain not only to the products, i.e. free RNA (I) and pyrophosphate (pp), but to all of the replication complexes also. This coherent nature of the autocatalytic (enzyme excess) phase is an essential requirement for discussing the kinetic behaviour analytically. (See below.)

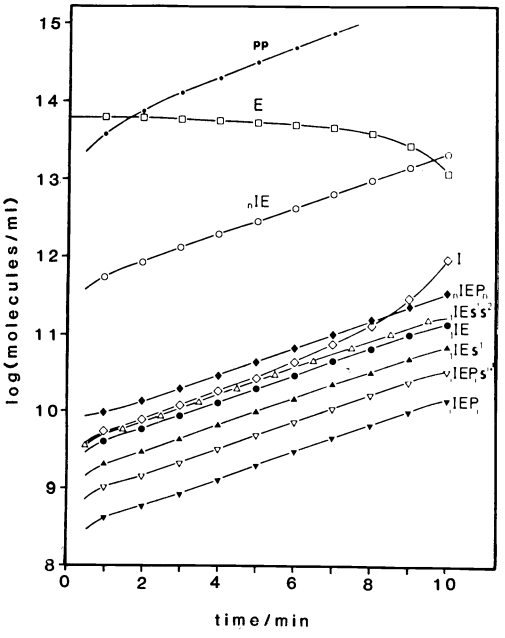

Fig. 4. Computer simulation of replication kinetics during the exponential growth phase. (For explanation of symbols and further discussion see Ref. 6) After a short induction phase in which steady-state concentrations of replication complexes are formed, a phase of coherent exponential growth begins. When the RNA concentration has grown to a level such that a significant fraction of the total enzyme is bound in replication complexes, the exponential growth phase goes over into a linear growth phase.

The exponential and linear growth phases can be followed in the laboratory by radioactive labelling of one or more of the nucleoside triphosphate monomers and measuring the radioactivity of the product after suitable separations. An example is shown in Fig. 5.

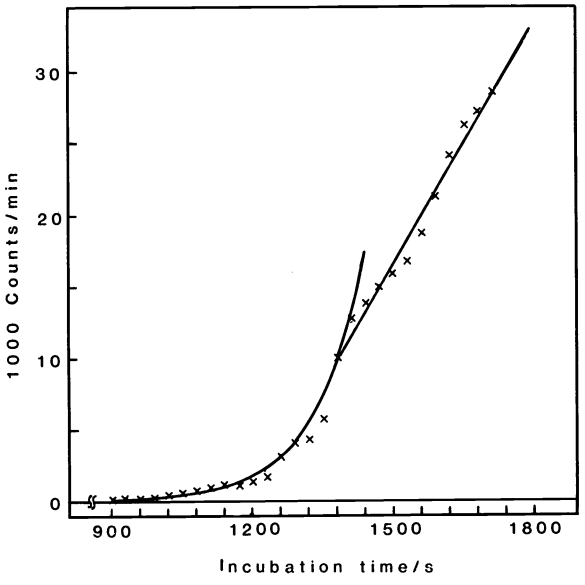

Fig. 5. Incorporation profile of radioactivity from (^{32}P)UTP in an experiment with Qβ replicase. The small hump in the incorporation rate at the transition from exponential to linear growth characterizes the onset of the requirement for template dissociation. For details see Ref. 5.

In place of measuring the total radioactivity incorporated into new RNA, different types of RNA, for example the plus and minus strands of a given RNA species or the double-stranded RNA formed when the single strands hybridize with one another, can be separated by suitable gel electrophoresis prior to the radioactivity measurement. In such experiments it is possible to distinguish the synthesis rates of individual RNA species. An example is shown in Fig. 6.

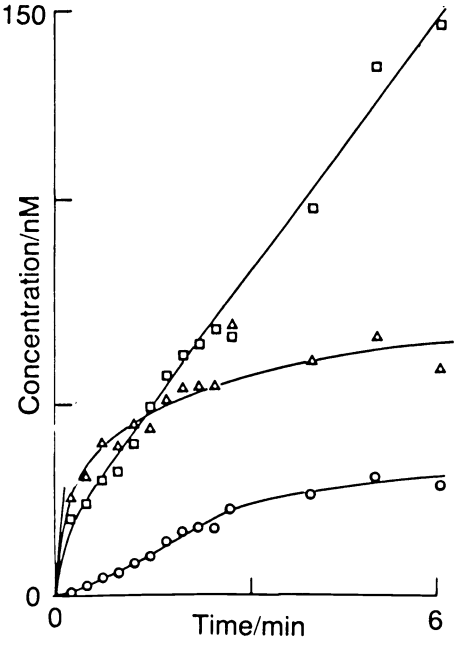

Fig. 6. Incorporation profiles of template RNA (circles), its complementary replica RNA (triangles), and the double-stranded RNA formed between them (squares). At long replication times, this particular RNA species achieves constant steady-state concentrations of single-stranded RNA and only the double-stranded RNA continues to grow in concentration.

The possibility of double strand formation has important implications for the outcome of the competition that ensues when more than one template is present in the incubation mixture. If double strand formation occurs only between the complementary partners, then it is possible for a coexistence to arise where both competitors share the enzyme and generate double strand in a constant ratio. If double strand can form between the two competitors also, then the stronger competitor drives the concentration of the weaker one to zero. A simulation illustrating this behaviour is presented in Fig. 7.

The final experimental aspect that will concern us in this paper is the kinetic analysis of the de novo RNA synthesis process. When template-free RNA synthesis was first observed, it was assumed that it resulted from evolutionary processes originating with impurity RNA carried from the replicase preparations. This was shown not to be the case by analysing the dependence of the phenomenon upon the concentrations of enzyme and substrate. An example of de novo RNA synthesis profiles is shown in Fig. 8. Details of the experiments are to be found in Refs. 1 and 5.

We can summarize the results of the experimental and computer simulation studies as follows. Replication of single-stranded RNA by Qβ replicase is a process involving two coupled replication cycles, one for each of the complementary RNA strands, each of which can be broken down into the basic steps of replication complex formation (template-enzyme binding) and initiation of replication, elongation and release of the replica strand, and reactivation of the enzyme by release of the 5'-bound template RNA.

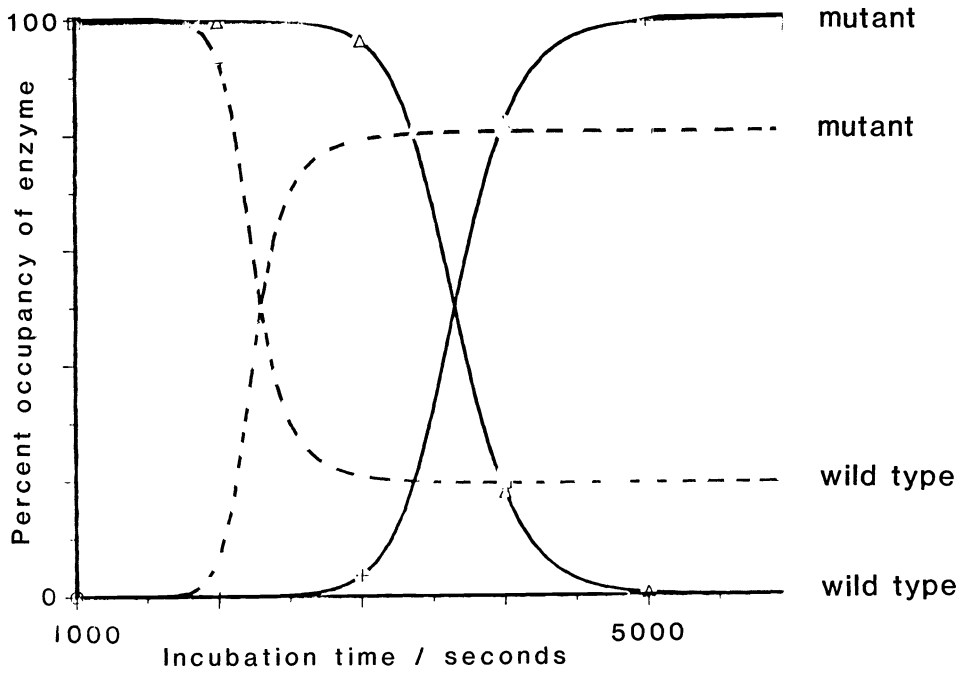

Fig. 7. Comparison of amounts of mutant and wild type RNA bound in replication complexes when double strand formation occurs only between complementary RNAs (dashed lines) and also between wild type and mutant RNAs (solid lines). (Combination of results presented in detail in Ref. 7)

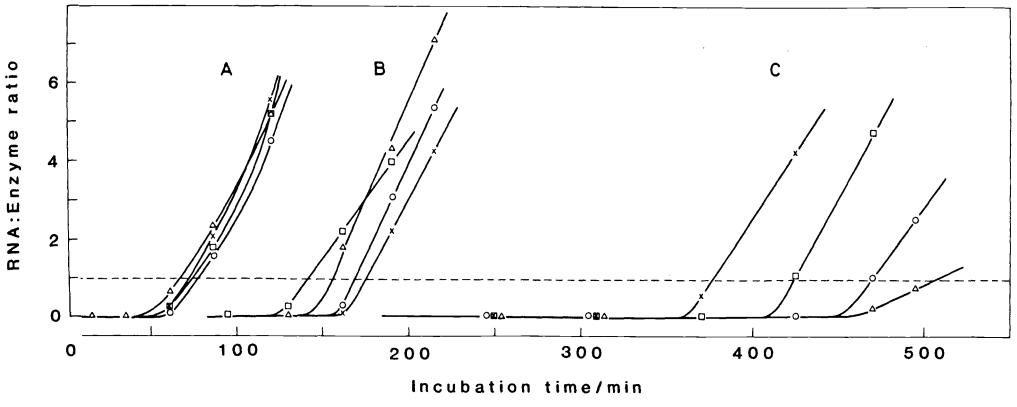

Fig. 8. RNA synthesis profiles for template-free incubations at three different (groups A,B, and C) nucleoside triphosphate monomer concentrations. The variability within each group shows that initiation of de novo synthesis depends on random molecular events. The concentration dependence shows that the initiation process involves participation of several monomer molecules, which is not the case for template-instructed kinetics. (After Ref. 5)

As important additional processes one has the inhibition of replication at high RNA concentrations by binding of RNA to the replica site (the reverse reaction of template release) and the formation of double stranded RNA from the initially formed single strands. Depending on the rate constant for double strand formation, the rate of single strand production eventually drops off to zero. The consequences of this mechanism for the synthesis profiles depend on the ratio of RNA to enzyme concentrations and upon the absolute value of the RNA concentration. At low RNA to enzyme ratios the RNA concentrations, both free and bound, grow exponentially in an autocatalytic

manner. When the RNA concentration equals or exceeds the enzyme concentration, the growth profile is linear until the RNA concentration attains a level where inhibition or double strand formation, or both, become appreciable. These characteristic phenomena, described here for a single RNA species, pertain in somewhat altered form to the situation where two or more different RNAs are present in the same incubation mixture. To describe the course of competition between different RNAs it is useful to express the kinetic behaviour in terms of analytical equations.

ANALYSIS OF REPLICATION KINETICS

In order to analyse the time evolution of the RNA concentration in an *in vitro* replication experiment the process shown in Fig. 2 must be expressed in the language of chemical kinetics by formulation of the corresponding set of kinetic equations. In the formal mechanism shown in Fig. 2 there are four steps for each cycle - initiation of replication (formation of the 3'-bound enzyme-template complex), elongation of the replica strand, release of the replica from the replication complex, and reactivation of the enzyme by release of the template. The detailed kinetic analysis (6,7) confirmed that replica release is so fast compared to the other steps that it can be combined with the elongation process to give a three step reaction mechanism, written in chemical equations as

$$I + E \xrightarrow{k_{A3}'} {}_1IE$$

$${}_1IE \xrightarrow{k_E} I' + {}_nIE$$

$${}_nIE \xrightarrow{k_{D5}'} E + I$$

In these equations the prefixed subscripts denote replication complexes with the template RNA (I) located at the first and n-th locations from the 3'-end and I' denotes the replica RNA. As template binding is essentially irreversible, its rate constant k_{A3}', characterizes the rate of initiation of replication. The single rate constant k_E characterizes the long sequence of steps in elongation and release of the replica strand, and k_{D5}', is the rate constant for the dissociation reaction of the 5'-bound template.

It is found experimentally that for most templates the rate constants for the complementary strands are about the same. This permits an important simplification of the kinetic equations, for then the two species I and I' can be considered the same and we can deal with four kinetic equations (for I, E, ${}_1IE$ and ${}_nIE$) rather than with seven (adding equations for I', ${}_1I'E$ and ${}_nI'E$). If double strand formation is to be included, then a kinetic equation for the reaction

$$I + I \xrightarrow{k_{ds}} I_2$$

must be added as well. The full set of differential equations to be considered is then

$$\frac{d[I]}{dt} = -k_{A3}'[E][I] + k_E[{}_1IE] + k_{D5}'[{}_nIE] - 2k_{ds}[I]^2$$

$$\frac{d[E]}{dt} = -k_{A3}'[E][I] + k_{D5}'[{}_nIE]$$

$$\frac{d[{}_1IE]}{dt} = k_{A3}'[E][I] - k_E[{}_1IE]$$

$$\frac{d[{}_nIE]}{dt} = k_E[{}_1IE] - k_{D5}'[{}_nIE]$$

$$\frac{d[I_2]}{dt} = k_{ds}[I]^2$$

Analysis of these equations can be carried out in detail. (6,7) Here we concentrate on some specific equations resulting from the analysis for two limiting situations, the exponential growth phase where the enzyme concentration [E] is constant, and the linear growth phase where the enzyme is saturated with template and there are no effects of product inhibition or double strand formation.

Exponential growth phase

At constant [E] all RNA-containing species grow in concentration with the common exponential growth constant

$$\kappa_+ = \frac{k_{A3'}[E] + k_E}{k_{A3'}[E]+k_E+k_{D5'}} \left\{ \left[\frac{k_{D5'}^2}{4} + \frac{k_{A3'}[E]k_E k_{D5'}(k_{A3'}[E]+k_E+k_{D5'})}{(k_{A3'}[E] + k_E)^2} \right]^{1/2} - 1 \right\}$$

For [E] values and rate constants typical of experimental conditions encountered in the laboratory this reduces to the high [E] form

$$\kappa_+ \dashrightarrow \frac{k_{D5'}}{2}\left[\left[1 + \frac{4k_E}{k_{D5'}}\right]^{1/2} - 1\right]$$

The enzyme concentration dependence is nearly linear on a Lineweaver-Burke plot, with an enzyme concentration at which the growth constant is half of its maximum value given by

$$[E]_{1/2} = \frac{k_E}{k_{A3'}}\left[\frac{1 + 2(k_{D5'}/k_E)^{1/2}}{3 - 2(k_{D5'}/k_E)^{1/2}}\right]$$

Linear growth phase

At enzyme saturation a partial steady state approximation can be applied by setting the derivatives of $[_1IE]$ and $[_nIE]$ equal to zero. The governing equations then become

$$\frac{d[I]}{dt} = \frac{v_{max}[I]}{K_{IE} + [I]} \qquad v_{max} = \frac{k_E k_{D5'}}{k_E + k_{D5'}}[E_o]$$

$$K_{IE} = \frac{k_E k_{D5'}}{k_{A3'}(k_E+k_{D5'})} \qquad [E] = \frac{K_{IE}[E_o]}{K_{IE} + [I]}$$

$$[E_o] = [E] + [_1IE] + [_nIE]$$

For the concentrations and rate constants of experimental interest $K_{IE} \ll [I]$ and $k_{D5'} \ll k_E$, for which the following limiting forms apply

$$\frac{d[I]}{dt} \dashrightarrow v_{max} \qquad [E] \dashrightarrow K_{IE}\frac{[E_o]}{[I]}$$

$$v_{max} \dashrightarrow k_{D5'}[E_o] \qquad K_{IE} \dashrightarrow k_{D5'}/k_{A3'}$$

These simple equations permit immediate insight into the criteria governing competition and selection between two RNAs present in the same incubation mixture. For example, if both RNAs have concentrations much less than the enzyme concentration, their competition is governed by the two κ_+ values and hence by the two $k_{D5'}$ and k_E values. If one of them (acting as a wild type) is in the linear growth range and the other at very small concentration, then the latter can grow if its relative concentration change κ_+ exceeds the

relative concentration change of the wild type, essentially $v_{max}/[I]$. Since κ_+ for such a low-concentration species may depend on [E], once the wild type RNA has attained such a high concentration that it drives [E] hyperbolically to very low values, exponential growth of competitors may be made impossible. On the other hand, in the linear growth range two competitor RNAs are subject to an entirely different selection criterion - only the two k_{A3}, values are rated in the selection process. If double strand formation is important for the wild type RNA, then the value of [E] is held at a steady-state value that provides a constant growth condition for competitors.

The reaction kinetics of RNA replication is a new form of biochemical kinetics. While the classic forms of steady-state and dynamic enzyme catalysis consider three fundamentally different species types - enzyme, substrate, and product - in their time evolution, the instructed synthesis of replication forces both the enzyme and the template into catalytic roles. Since the RNA acts both as catalyst and as reaction product, essentially new kinetic forms arise, giving autocatalytic behavior when a single RNA template is present and a broad variety of different forms of competition and selection when two or more are present. The course of the molecular evolution does depend on the contraints imposed by selection of conditions - whether the system is closed or open (either as a flow reactor or in serial dilution experiments), whether the RNA concentration is high enough for double strand formation to occur, whether selection pressure is imposed by the environmental conditions such as salt concentration or intercalating species such as ethidium bromide that affect the stabilities of tertiary RNA structures. For our further development here the exact course that competition and selection take is not important. What is important is to recognize that Qβ replicase provides a laboratory experiment in which the process of self-replication occurs in a way that is quantitatively understandable and can be utilized to study the process of molecular evolution.

We turn now to the application of RNA replication for the study of molecular evolution in a controlled manner.

EVOLUTION REACTOR

One of the key developments in the history of mathematics was the proposal by Alan Turing in 1936 of a computing machine for finding the values of mathematical functions. Today his ideas are often cited as forerunners of the concepts behind modern digital computers. Actually, Turing's intentions were entirely different. His computing machine was designed to test a specific mathematical hypothesis, namely whether any given mathematical function is computable in a finite number of operations. It did to be sure take the form of a real machine, with a moving tape that could be used to record information generated by the machine, and in this respect there a many analogies to modern digital computers. Turing's purpose, however, was to test a principle, not to design a computer. In this respect one sees a close resemblance between Turing's computing machine and the Carnot engine which was utilized as a working concept in the 19th century to develop the theory of heat engines, and is well known today as a pedagogical device for explaining the equations of thermodynamics to university students. No one would set out to build a heat engine using the sequence of steps comprising the Carnot cycle, but at the same time no introductory textbook on thermodynamics sets out to develop the equations describing the fraction of heat that can be converted to work without the Carnot cycle. Turing's computing machine and Carnot's heat engine both have as their main purpose the clear formulation of basic abstract concepts.

The idea of a machine to test biological concepts was already proposed in 1966 by von Neumann. (12) This was well before there was a clear picture of the biochemical basis of genetics, however, and now we can formulate an appropriate machine much more concretely, even to the point of realizing it in the laboratory. Our task can be formulated in three central theses -

(1) All known forms of life are complex in form and function.

(2) The complexity is organized at the molecular level - Cf. the 'aperiodic crystals' of Schrodinger. (13)

(3) This organization enables optimization of all characteristics of the organism that are required for its survival and development.

If the basic concepts addressed by the Turing and Carnot machines are 'computability' and 'efficiency', then the concept addressed by the analogous machine of biology is 'optimizability', or more precisely the 'functional optimizability of complex structures'. Darwin's insights show us that the basis of optimization is self-reproduction. Self-reproduction leads to selection, selection under a finite mutation rate leads to evolution, and evolution leads to the optimal structure.

The Darwinian principle of selection implies that a correlation exists between a population space - survival expressing itself in relative population numbers - and a value space in which individual sequences are rated according to their stability and replicability. Of course, it is upon the quantitative nature of this correlation that the concept of the machine must be based. This quantitative relationship has been developed in a number of studies, with quite unexpected results. Contrary to the general belief that selection is deterministic only after appearance of some advantageous mutant, which upon its appearance begins to grow exponentially in population, it was found that the very appearance of successful mutants is guided by the topology of the value space through the topology of the population space. This is to say that the successful mutant usually reveals itself as an offspring of a chain of precursor mutants that provide a 'ridge' connection between the peak (in both value and population space) of the current wild type and the new (higher) peak of the advantageous mutant. The mutant may differ genetically from the wild type at many points in its gene, i.e. may have different occupation at many base locations, corresponding to a long ridge in the value space. The theory shows that mutants belonging to such ridges are heavily overpopulated compared to mutants belonging in the 'valleys' or 'plains' elsewhere in value space. The fact that peaks in value space are clustered in certain regions - like mountain ranges in terrestrial topography - provides guidance for producing useful mutants in quantity. A quantitative analysis shows that sequences with lengths that are suitable for single genes can indeed be optimized by motion along value ridges with error rates corresponding to Qβ replicase and evolution times corresponding to laboratory experiments.

These results provide favourable promise for the actual construction of a machine . Its principle of operation is new - instead of trying to screen a very large number of mutants in the hope of finding advantageous offspring, one may sample the mutant distribution of say only a thousand members and probe their value topology. This value topology - explored in an iterative procedure, just as one would go about exploring the surface of a planet - furnishes guidance about the best direction to continue the search, namely in the direction of highest peak density. A prerequisite of the procedure is to keep track of the branched genealogies during the successive generations of mutant spectra. The summary of our experience with RNA self-reproduction is that their optimal structures do evolve according to such a 'Darwinian logic'. (See especially Ref. 10) We can formulate the operation of this Darwinian logic as a procedure

10 START WITH SELECTED GENOTYPE
20 LET IT REPRODUCE, MUTATING OCCASIONALLY
30 FORCE DIFFERENT GENOTYPES TO COMPETE
40 NATURAL SELECTION OF QUASI-SPECIES AROUND
BEST-ADAPTED GENOTYPE OCCURS
50 WHEN ADVANTAGEOUS MUTANT APPEARS - GOTO 10

This procedure is of course not intended to be realized on a digital computer (although it has been, see Ref. 14) but rather as an iterative sequence of chemical steps. For the case that 'best-adapted' refers to the self-replication rate only, as in the experiments done so far and described by the analysis given above, this iterative sequence proceeds automatically as long as the system is open, i.e. as long as a fresh supply of monomer (or monomer and enzyme, if selection in the exponential growth phase is to be carried out) is provided.

In this procedure the evolving property is required to be expressed in the replication rates, through which selection of the self-replicating entity is effected. If one wants to optimize a single protein molecule with a particular (enzymic) property, then this phenotypic advantage has to contribute to the selection value of its genotype, otherwise selection is not possible. While in nature the feedback from phenotype to genotype is effected through the survivability and replication efficiency of the whole organism, such a procedure will not work for isolated genes, because phenotypic advantages of the translation products do not express themselves in terms of replication rates of these specific genes. Artificial selection of isolated products is still possible, however, by using a screening test for the desired property. (An analogy would be the screening test that an agronomist might use to breed strawberries that ripen particularly late in the season.) Such a test would require cloning of single mutants and screening all the clones in order to identify advantageous mutants. Locating an advantageous mutant directly by such a test probably could not be compressed to a laboratory time scale even if the dimension scale could be attained. The theory suggests, however, that artificial selection may be fast if it is guided along ridges between value peaks, as in natural selection. Hence the alternative method of designing a molecular evolution machine is to establish the value landscape of the mutant spectrum rather than looking for advantageous mutants. In order to do this practically one must know the mutation distances within the mutant spectrum. The average mutant distances can be established only if the mutant spectrum is produced through a hierarchical, serial cloning procedure to provide tree-like genealogies. Identification of value peaks then proceeds by iteration and narrowing down the procedure of topological evaluation.

Controlled optimization by the above procedure really becomes an interesting challenge when genotype and phenotype are different molecules.

Let us therefore expand the procedure as follows -

```
10 PRODUCE A MUTANT SPECTRUM OF SELF-REPRODUCING TEMPLATES
20 SEPARATE AND CLONE INDIVIDUAL MUTANTS
30 AMPLIFY CLONES
40 EXPRESS CLONES
50 TEST FOR OPTIMAL PHENOTYPES
60 IDENTIFY OPTIMAL GENOTYPES
70 RETURN TO 10 WITH A SAMPLE OF OPTIMAL GENOTYPES
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Many additional questions present themselves when one faces the practical challenge of constructing a machine to execute this procedure. In view of the large numbers of clones and the multiplicity of iterations, it is obvious that automatic operation is required. The clones have to be addressable, the analytical methods must combine parallel processing and automatic sampling with sensitivity and speed. With such elaboration and scale, experimental biology might well become 'Big Science'. Which ought not to be surprizing, as the underlying physical problem of biology is indeed 'complexity and its reproducibility'.

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