BIOCHIMIE, 1975, 57, 587-595.

Kinetic amplification of enzyme discrimination.

Jacques NINIO ◇ .

The Salk Institute for Biological Studies P.O. Box 1809, San Diego, California 92112.

(12-12-1974).

Summary — The dependance of the accuracy of enzymatic systems on the mechanism of the catalyzed reaction is investigated, using a probabilistic approach. Certain mechanisms of reaction, involving a delay in one of the steps act as kinetic amplifiers of molecular discriminations. The relationship between our scheme for a delayed reaction [1] and Hopfield's scheme [2] is discussed.

INTRODUCTION.

Many enzyme systems discriminate with remarkable efficiency between their substrate and closely related molecules. It is generally accepted that this discrimination depends in large measure on an accurate matching of the shape of the enzyme to that of the substrate either during binding of the ligand or at a subsequent step of the reaction [3, 4].

The examination of recent results (to be discussed later) on the accuracy of DNA polymerization, on ribosomal selection and on the discriminative abilities of the aminoacyl-tRNA ligases led us to modify that point of view, at least for certain classes of enzymatic systems. Our picture involves two elements :

a) An enzyme has certain discriminative abilities by virtue of its matching to the substrate. This may be reflected through a difference in the dissociation constants k_d^S and k_d^A of the enzyme-substrate and the enzyme-analogue associations, or through differences in other kinetic parameters of the reaction.

b) The enzymatic reaction is considered as a processing of the substrate and the analogue leading to products P^S and P^A. The process as a whole contains one (or more) discriminative step, and takes more or less adavantage of the discriminative potentialities reflected in the ratio k_d^A/k_d^S.

Our examination of several cases show in agreement with intuition, that in most cases, the discrimination of the whole process is smaller than that of the discriminative step. Thus, if the concentrations of substrate and analogue are equal, we have in general $[P^S]/[P^A] \leq k_d^A/k_d^S$. However, we looked for and found a class of reactions which allow the process as a whole to be more discriminative than its discriminative step. We are able to construct schemes for which $[P^S]/[P^A]$ may approach $(k_d^A/k_d^S)^2$ or even higher powers of the ratio of the two dissociation constants (*).

Thus, in principle, Nature has at least two strategies for achieving accuracy. It may design a very sophisticated binding site, or it may select a shrewd mechanism of reaction which takes advantage of small differences in kinetic parameters. The second strategy would appear more interesting in situations where the enzyme system needs to solve *several* accuracy problems. This is precisely the case encountered in DNA polymerization and in ribosomal selection of tRNA.

DNA polymerization. A same enzyme is involved in the replication of each of the four bases A, T, G, C. dCTP which is a substrate in one case (when a G is copied) becomes a dangerous analogue with respect to the three other cases.

Mutants of phage T4 DNA polymerase have been obtained, displaying either mutator [5] or antimutator [6] properties. These mutants have been extensively characterized both genetically [7-9] and biochemically [10-13]. The interesting

◇ Present address where all correspondence should be addressed: Institut de Biologie Moléculaire, Tour 43, 2 Place Jussieu, Paris (5e).

(*) Our findings were briefly presented at « Ecole d'Eté sur l'Evolution des Macromolécules Biologiques » (Roscoff, May 1974) and are discussed in the proceedings of that meeting [1]. After the submission of this more complete paper, John Hopfield's article bearing on the same subject [2] reached us, and the manuscript was revised in order to include a comparison between the two approaches and discuss recent experimental findings.

feature is that different point mutations in the DNA polymerase gene have the effect of lowering or raising simultaneously most of the error-levels that can be measured : spontaneous A.T. ⟶ G.C or G.C ⟶ A.T transitions, transversions, errors due to the incorporation of base-analogues such as 2-amino purine or bromo-U, etc. [9]. Yet, the aminoacid substitutions resulting from the mutations should have different geometrical consequences.

This suggests that T4 DNA polymerase makes use of a discriminative procedure which can be applied with more or less vigour. More precisely, it was proposed that the kinetic balance between two steps of the reaction (polymerizing activity versus exonuclease activity) was more important for DNA polymerase selectivity than the discriminative potentialities of the individual steps [13]. Unfortunately, the authors reached that conclusion on the basis of a kinetic treatment which is actually irrelevant (see the Discussion).

Ribosomal selection. Here again, a number of ribosomal mutants are known which have the effect of changing in a parallel maner « natural errors », and all the levels of nonsense and missense suppression [14-16]. Judged from the point of view of the geometry of the codon-anticodon interactions, the various effects appeared contradictory [17]. However, they could be reduced to a single effect when geometry considerations were abandonned. They could be accounted for by a change in the discriminative abilities of the ribosome [18] explained by the change of a single kinetic parameter in the reaction [19].

UNDELAYED REACTIONS.

We start our study of the discriminative « shrewdness » of various reaction schemes with the classical Michaelis model :

$$E + S \underset{k_{-1}^S}{\overset{k_1^S}{\rightleftarrows}} ES \xrightarrow{k_2^S} E + P^S \qquad (1)$$

We assume throughout the article that such macroscopic representations are exact, i.e., they reflect accurately the processes at the microscopic level. Then the kinetics can be expressed in terms of probabilities. $[S]\ k_1^S\ dt$ is the probability dp that a free enzyme molecule will form an enzyme-substrate complex in the infinitesimal time dt, with similar expression for k_1^S and k_{-1}^S. When the complex ES is formed, it disappears exponentially with time according to $\exp(-k_{-1}^S t - k_2^S t)$. k_{-1}^S is the reciprocal of the mean sticking time $\bar{t}^S$ of the complex, that would be measured in the absence of the product-forming reaction.

Once an enzyme-substrate or an enzyme-analogue association is formed, however transitory it may be, what is the probability of completion of the reaction ? For any short interval of time dt, a fraction k_{-1}^S decays, while a fraction k_2^S dt leads to product formation. Thus, the average probability of completion of the reaction is quite simply given by $P = k_2^S/(k_2^S + k_{-1}^S)$ and by the equivalent expression for the analogue A. If we compare the rates at which substrate and analogue are transformed, we have the ratio :

$$\frac{V^S}{V^A} = \frac{[S]\ k_1^S\ P^S}{[A]\ k_1^A\ P^A} \qquad (2)$$

We will define the discrimination term D as the velocity ratio of Eq (2) divided by the concentration ratio :

$$D = \frac{V^S}{[S]} \Big/ \frac{V^A}{[A]} \qquad (3)$$

In the case of Michaelis kinetics, D is a product of two terms : the ratio of the collision efficiencies of substrate and analogue, and the ratio of the probabilities of completion of the reaction after a collision :

$$D = \frac{k_1^S}{k_1^A}\ \frac{P^S}{P^A} \qquad (4)$$

This expression can be extended to any other mechanism of reaction for which there is only one conformational state of the enzyme allowing an entry of the ligands.

Consider the special case where $k_1^S = k_1^A$ and $k_2^S = k_2^A = k_2$. When the reaction time $1/k_2$ is very short compared to both sticking times $1/k_{-1}^S$ and $1/k_{-1}^A$, $D = 1$. The enzyme does not discriminate between substrate and analogue. As the reaction time increases with respect to the sticking times, discrimination also increases and reaches the limit $D = k_{-1}^A/k_{-1}^S = \bar{t}^S/\bar{t}^A$ for infinite reaction times. Thus, when the only kinetic difference which is exploited by the enzyme is that of the sticking times, discrimination is at best in the ratio of the sticking times. (Of course, a larger D value can be obtainer if $k_2^S > k_2^A$).

The result can be generalized to the case where there are additional intermediate stages in the formation of the product :

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES_{(1)} \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} \cdots\cdots \underset{k_{-n}}{\overset{k_n}{\rightleftarrows}} ES_{(n)} \xrightarrow{k_{n+1}} E + P^S \qquad (5)$$

When the complex $ES_{(1)}$ is formed, it may either dissociate, or yield product with a probability P which is given by the elegant formula of Knorre and Malyguine [20].

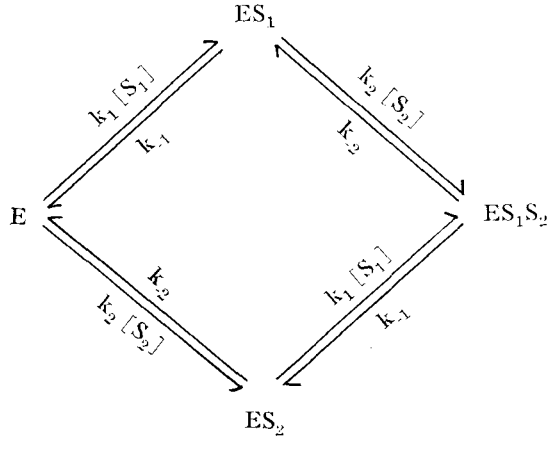
$$\frac{I}{P} = I + \frac{k_{-1}}{k_2}\left\{I + \frac{k_{-2}}{k_3}\left[I + \ldots + \frac{k_{-(n-1)}}{k_n}\left(I + \frac{k_n}{k_{n+1}}\right)\right]\right\} \quad (6)$$

From there, it is easy to show that when all the kinetic parameters of substrate and analogue save one pair (k_i^S and k_i^A or k_{-i}^S and k_{-i}^A) are equal, the discrimination cannot be better than the ratio of the kinetic parameters : k_i^A/k_i^S or k_{-i}^A/k_{-i}^S. The result applies to any pair of kinetic parameters, and to a reaction involving any number of steps.

DELAYED REACTIONS.

In this section we consider the case where binding is the discriminative step. We could have said, for a Michaelis scheme, that the enzyme is « comparing » the sticking times $\bar{t}^S$ and $\bar{t}^A$. Suppose now that the reaction subsequent to binding of the substrate or the analogue is delayed by a time t_0. The times during which the reactions may occur are now $\bar{t}'^S = \bar{t}^S - t_0$ and $\bar{t}'^A = \bar{t}^A - t_0$. It is clear that the ratio $\bar{t}'^S/\bar{t}'^A$ of these apparent sticking times can be considerably larger than the ratio t^S/t^A. This raises the question : are such delayed reactions conceivable chemically ? At first, one may think that the realization of delays should involve effects which are outside the range of validity of the traditional chemical notation. It turns out however that delays can be obtained by sequences of conventional chemical events. Then, t_0 is not a fixed time, but a random variable with a mean value that can be computed.

The following is the simplest example of delayed reactions we have been able to construct. We start with a two-substrate reaction in which the binding of one substrate has no influence on the binding of the other substrate (see Eq. (7)).



$$\xrightarrow{k_3} \quad E + P \quad (7)$$

and we make the $E \rightleftarrows ES_2$ branch of the reaction irreversible by coupling it to a very rapid destructive process, for instance :

$$ES_2 + ATP \xrightarrow{\text{(very rapid)}} E + S_2 + AMP + PP \quad (8)$$

or, if S_2 *is* ATP, this last reaction can be replaced by the *in situ* degradation of ATP.

The reason for the additional (and essential) reaction will be made intuitive later.

Let us introduce the various probabilities of the direct transitions from one state to another :

$p = k_2[S_2]/(k_2[S_2] + k_{-1})$ for $ES_1 \longrightarrow ES_1S_2$
$q = k_3/(k_3 + k_{-2} + k_{-1})$ for $ES_1S_2 \longrightarrow P$, and
$r = k_{-2}/(k_3 + k_{-2} + k_{-1})$ for $ES_1S_2 \longrightarrow ES_1$

Again we ask the question : once ES_1 is formed, what is the probability that it will lead to P ? We may have several back and forth motions from ES_1S_2 to ES_1 and ES_1 to ES_1S_2 before forming the product. Thus, the overall probability is given as the sum of the series :

$$P = pq + prpq + (pr)^2pq + \ldots\ldots \quad (9)$$

$$P = \frac{pq}{1 - pr} = \frac{k_2 k_3 [S_2]}{k_2 k_3 [S_2] + k_{-1}(k_3 + k_{-2} + k_2[S_2]) + k_{-1}^2} \quad (10)$$

The interesting feature is the presence of a quadratic k_{-1}^2 term. For very short sticking times (large values of k_{-1}), and if the only kinetic difference which is exploited is that of sticking times, we have :

$$D \simeq (\bar{t}^S/\bar{t}^A)^2 \qquad (11)$$

Thus, analogues with very weak binding constants are processed according to the squares of the sticking times.

In order to determine more precisely the domain of validity of the quadratic effect, one has to take into account the two branches of the reaction. Let us call α the concentration of E relative to ES_2 : $\alpha = [E]/([E] + [ES_2])$ — α can be computed through the steady — state equations. The complete expression for P is :

$$P = \alpha \frac{pq}{I\text{-}pr} + (I\text{-}\alpha) \frac{q}{I\text{-}pr} \qquad (12)$$

The quadratic effect is found in the pq/(I-pr) term, and not in the q/(I-pr) term. The irreversible coupling of Equation (8) has the effect of making α close to one and thus is a necessary condition for the validity of Equation (10). The quadratic effect requires a large value of k_{-1} (superior to k_3, k_{-2} and k_2 $[S_2]$) but not too large a value either, for then the (I-α) q/(I-pr) term would cease to be negligible compared to the αpq/(I-pr) term. Thus, the quality of the irreversible coupling (reflected in the value of α) determines how far in the domain of short sticking times, the amplification effect may hold.

The quadratic effect is absent when the two-step reaction follows a mechanism of strictly ordered addition such as :

$$E + S_1 \underset{k_1}{\overset{k_{-1}}{\rightleftarrows}} ES_1 \qquad ES_1 + S_2 \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} ES_1S_2 \xrightarrow{k_3} E + P \qquad (13)$$

This reaction can be rewritten as :

$$E + S_1 \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES_1 \underset{k_{-2}}{\overset{k_2\,[S_2]}{\rightleftarrows}} ES_1S_2 \xrightarrow{k_3} E + P \qquad (14)$$

and the discrimination can be obtained from Equation (6).

Intuitive explanation of the delayed reaction effect. Let us consider that the complex ES_1 has been formed at time 0, and let us follow the evolution of the enzyme-substrate complex with time. Initially, S_2 is absent from the complex : its probability of presence is zero. S_2 may come, leave, come again, etc. Its probability of presence rises gradually until it reaches a maximum level corresponding to equilibration (fig. 1). Suppose we divide the time-axis in short successive intervals of duration δt. The sticking times of substrate and analogue can be written as $N^S\,\delta t$ and $N^A\,\delta t$ respectively, where N^S and N^A can be taken as integers.

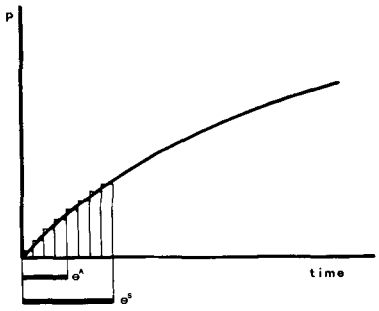


FIG. 1. — *Intuitive explanation of the kinetic amplification effect.*

We consider a two-substrates reaction (Equations (7) and (8)). The first substrate binds to the enzyme with a sticking time θ^S larger than the sticking time θ^A of the analogue A. We take as origin of the time-axis the instant when the complex ES_1 or EA is formed. The probability of presence of S_2 on the enzyme (P, in ordinate) increases with time. The asymptotic value of P corresponding to equilibration between arrivals and departures of S_2 is $k_2\,[S_2]/(k_c\,[S_2] + k_{-2})$. The overall probability of the reaction when S_1 or A is on the enzyme can be obtained by adding the probabilities of the reaction during consecutive intervals of time. For every small interval of time δt, the probability of the reaction is the product of the probability of presence of S_2 (the value of P taken on the curve) by a constant $\beta\delta t$. $\beta\delta t$ is the probability of forming a product during a small interval of time δt when both S_1 and S_2 are on the enzyme. Thus, the overall probabilities corresponding to sticking time θ^S and θ^A are proportional to the *areas* under the curve. For small θ's they vary as the squares of sticking times.

During an interval of time δt, and provided both S_2 and S_1 are present on the enzyme, there is a probability δp of making the product. Initially, the probability of presence of S_2 is low and what happens makes only a small contribution to the total probability of forming the product.

The ratio of the two probabilities for substrate and analogue (if their sticking times are short enough) can be approximated by (see Fig. 1) :

$$P^S/P^A = (1+2+3+\ldots+N^S)\,\delta p/(1+2+3+\ldots+N^A)\delta p \simeq (N^S/N^A)^2 \qquad (15)$$

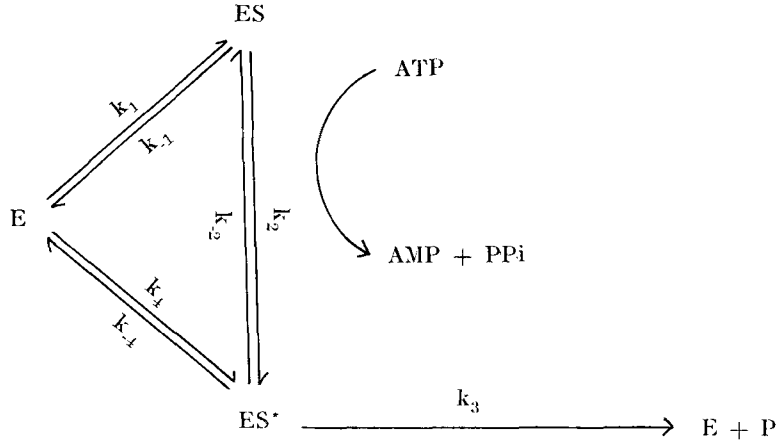
(This was indeed the intuitive consideration which led us to the construction of the reaction scheme).

The preceding probabilistic description does not apply to strictly ordered reactions. For then, upon binding of S_2, S_1 is not allowed to leave the complex prior to the departure of S_2, and the sticking time of S_1 cannot be defined as an entity independant of the binding of S_2.

Thus, the probabilistic model implied random departures for S_1 and S_2. If this condition is realized, the principle of microscopic reversibility requires that the order of binding of S_1 and S_2 be also random, and we are led to scheme (7). However, we are interested in the upper branch of the reaction, exclusively. The lower branch can discriminate at best in the ratio of sticking times. From there, the necessity of Equation (8) follows naturally.

COMPARISON WITH HOPFIELD'S SCHEME.

Hopfield's scheme can be written as :



(16)

Let us introduce the elementary probabilities of transition :

$p = k_2/(k_2 + k_{-1})$ for $ES \longrightarrow ES^*$
$q = k_3/(k_3 + k_{-2} + k_{-4})$ for $ES^* \longrightarrow P$
$r = k_{-2}/(k_3 + k_{-2} + k_{-4})$ for $ES^* \longrightarrow ES$

When the substrate binds to E, it may go either to ES with the frequency $\alpha = k_1/(k_1 + k_4)$ or to ES* with the frequency I-α. Thus, the overall probability of forming the product after a collision between the enzyme and the substrate is :

$$P = \alpha \frac{pq}{I\text{-}pr} + (I\text{-}\alpha) \frac{q}{I\text{-}pr} \qquad (17)$$

Kinetic amplification is obtained as in our example through the pq/(I-pr) term. There is a complete mathematical analogy between the two schemes.

In both schemes, there are two gates of exit for the substrate, allowing a double-check mechanism. Since the substrate could bypass the double-check if entering directly through the second gate, an irreversible energetic coupling is introduced in both schemes. It has the effect of making the second gate practically inaccessible for substrate entry. The main difference between the two schemes concerns the positionning of the irreversibility with respect to the last step of the reaction.

Physically, while our scheme corresponds to an authentic time-delay, Hopfield's scheme corresponds to a pumping effect analogous to the Overhauser effect in N.M.R., and was discovered through this analogy (Hopfield, personal communication).

DELAYED ESCAPE OF THE PRODUCT.

In the preceding example binding was the discriminative step. We present now a scheme wherein the amplification effect is exerted upon a step of a different nature. The model is directly inspired by known biological facts.

Some DNA polymerases [11 12, 21, 22] and some aminoacyl-tRNA ligases [23, 24] are known to possess a hydrolytic activity towards the product of their reaction, which is independant of their synthetic activity (the latter being driven by the splitting of a pyrophosphate bond). Consider an isoleucyl-tRNA ligase which has just aminoacylated a molecule of $tRNA^{Ile}$ with either isoleucine or valine. While the acylated tRNA is still on the enzyme it is subjected to a hydrolytic activity with rates k^S and k^A for Ile-$tRNA^{Ile}$ and val-$tRNA^{Ile}$ respectively. Let us call t the time that the loaded tRNA spends on the enzyme. The probabilities of hydrolysis for the two molecules are I-exp(-k^St) and I-exp(-k^At) respectively. It is

easy to show from there that the ratio of hydrolyzed Val-tRNA^{Ile} to hydrolyzed Ile-tRNA^{Ile} cannot exceed k^A/k^S. Howver, this k^A/k^S ratio is not the relevant one. What is really important is the ratio of the *surviving* molecules of the two kinds. Then, the answer is quite different. If the ratio of correct to incorrect molecules bound to the enzyme before the hydrolytic step is p^S/p^A, it becomes after that step :

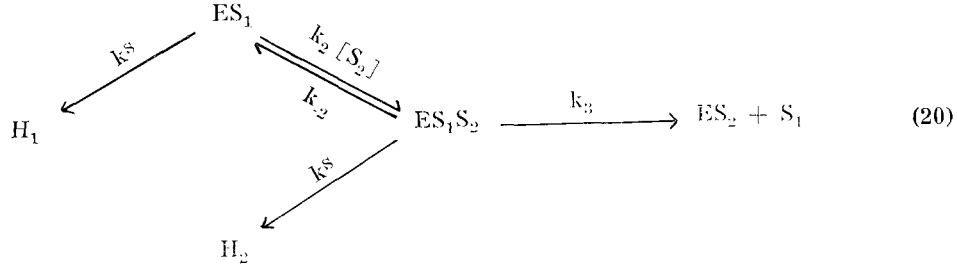
$$P^S/P^A = p^S/p^A \, e^{-(k^S-k^A)t} \qquad (18)$$

This suggests the possibility that an enzyme may achieve an exponential exploitation of differences in kinetic parameters. Here, we reasonned as if the time t available for the hydrolysis of the product was a fixed time. This condition is not realized in the most simple situation, when the product leaves the enzyme according to first-order kinetics with a rate-constant k. We would obtain :

$$P^S/P^A = p^S/p^A . (k + k^A)/(k + k^S) \qquad (19)$$

and the contribution of the proof-reading function to discrimination would not be better than the ratio k^A/k^S.

However, if the escape of the product is delayed, although t remains a random variable, its distribution is more stepwise and one obtains an amplification effect. As a practical way of achieving a (random) delay, consider the following model. Upon aminoacylation, the tRNA is firmly hooked to the enzyme. Its departure requires a conformational change of the tRNA, or of the enzyme, or the binding of another molecule to the enzyme, for instance the binding of a second amino acid [26, 27]. The overall situation can be described by the scheme :



(20)

where S_1 represents the acylated tRNA, S_2 stands for the second aminoacid and the H's refer to states of the enzyme immediatly after the occurence of a hydrolysis. (We are not interested here in knowing what are the substrates that remain temporarily on the enzyme after hydrolysis). The situation presents a formal similarity to that of the preceding section. When ES_1 is formed, the probability of escaping hydrolysis is obtained replacing in Equation (10) k_{-1} by k^S. Now, the amplification effect bears on the hydrolysis rates, and not on sticking times.

THE RANDOM WALK OF A DNA POLYMERASE.

The situation with the bifunctional DNA polymerases presents additional interesting features. After the template-directed incorporation of a base (n) the enzyme moves one step forward for the incorporation of the next base (n + 1). Meanwhile, the base incorporated last is « tested » by the exonucleolytic function. If hydrolysis occurs, the enzyme moves one step backwards. On the other hand, the incorporation of the base (n + 1) by the head of the enzyme, with the subsequent forward motion eventually allows the base (n) to escape from the danger of exonucleolytic attack.

When S is incorporated, there is a probability q^S of going backwards (hydrolysis) and a probability $I-q^S$ of moving forward (incorporation). In case of incorporation S or A are introduced with respective probabilities p^S and p^A ($p^S + p^A = I$). We suppose for simplicity that the enzyme is replicating a homopolymer and that p^S and p^A are independant of the preceding base. Let us call P^S and P^A the overall frequencies of incorporation of S and A in the newly synthesized strand. In the case of the hydrolytic function of the aminoacyl-tRNA ligase we had :

$$P^S/P^A = p^S (I-q^S)/[p^A (I-q^A)] \qquad (21)$$

Now, the expression is more complicated since the polymerase can move a number of steps forward, go backward several steps, etc. We have to consider all possible trajectories of the DNA polymerase along the template. We have started studying the problem both experimentally and theoretically (J. N. & F. Bernardi, work in progress). Our treatment leads to the following expression :

$$P^S = p^S (I-q^S)/[I-K(I-q^S) \quad (22)$$

where K is the small root of Equation (23) :

$$K^2 (I-q^S) (I-q^A) - K (I-q^S q^A) + p^S q^S + p^A q^A = 0 \quad (23)$$

Setting :

$$\alpha_A^S = [I-K(I-q^A)]/[I-K(I-q^S)] \quad (24)$$

one gets

$$P^S/P^A = \alpha_A^S \, p^S(I-q^S)/[p^A(I-q^A)] \quad (25)$$

α_A^S appears as a corrective factor which may be called the « peelback » correction term [28]. Take the limiting case where $p^S = q^S = I-p^A = I-q^A$, one obtains :

$$\alpha_A^S = q^A/(I-q^A) \quad (26)$$

Therefore one can make the peelback term as important as one wishes. However, in most practical situations, the peelback term is rather unimportant, and will be neglected in the forthcoming considerations.

The elementary probabilities p's and q's are related to the elementary kinetic parameters (the k's). Suppose that k^S and k^A are the kinetic constants for the hydrolysis of a correct base-pair and the corresponding incorrect base-pair as measured in the absence of synthesis. The contribution of the hydrolytic function to discrimination $(I-q^S)/(I-q^A)$ may be larger than k^A/k^S only if there is a delay in the escape from the exonucleolytic site. Whether or not a delayed escape effect holds would depend on the mechanism of incorporation at the head of the enzyme ; that is on the number of reversible or irreversible steps involved and the balance between the kinetic parameters of the propagation reaction and those of the hydrolytic reaction.

Discrimination being related to the time which separates two incorporations, it should depend on the concentrations of the dNTP's. More precisely, the errors of incorporation at any position along the chain would increase with *increasing* concentrations of that dNTP which is to be incorporated at the *next* position. Such an effect can in principle be revealed in classically designed experiments [29, 30].

Practically, the exonuclease activity results in the conversion of nucleoside triphosphates into nucleoside monophosphates. If Hopfield's scheme applies to DNA polymerization, one should also be able to observe a release of nucleoside monophosphate, but governed by different laws than the release due to the exonuclease activity. One may also wonder why Hopfield's scheme could not be applied twice, the polymerase cleaving two phosphates in a row. A consequence of a repeated Hopfield scheme for DNA polymerization would be the formation of both nucleoside diphosphates and nucleoside monophosphates in the course of the polymerization reaction. We have performed a number of experiments with *E. coli* DNA polymerase I, *E. coli* RNA polymerase and two eukaryotic DNA polymerases (J. N., F. Bernardi, G. Brun, A. Assairi, M. Lauber and F. Chapeville, manuscipt in preparation). In most cases, we are able to detect a release of nucleoside diphosphate and nucleoside monophosphate which parallels the incorporation reaction. For instance, using *E. coli* DNA polymerase I, Poly (dC) as a template in the presence of manganese, dGTP and dATP as competing substrates, we followed in parallel experiments dGMP incorporation and dGDP and dGMP release and also dAMP misincorporation and dADP and dAMP release. We observed a perfect parallelism between dGTP consumption (resulting mainly in dGMP incorporation) and dATP consumption (mainly converted into dADP). By « parallelism », we mean that the condition Log ([dATP (t)]/[dATP (O)])/Log ([dGTP (t)]/[dGTP (O)]) = constant held true throughout the reaction. Furthermore, the constancy of the ratio [dADP]/([dAMP incorporated] + [dAMP released]) was also verified to a very good approximation. This and other evidence suggest that nucleoside incorporation and conversion of nucleoside triphosphate into nucleoside diphosphate or monophosphate are alternative outcomes of the interaction of a nucleoside triphosphate with the polymerase-template complex in the course of polymerization. They do not prove yet that Hopfield's incorporation scheme is correct. The possibility remains that nucleoside mono and diphosphate formation are due to a parasitic abortive branch of the polymerization reaction.

DISCUSSION.

The probabilistic approach that we have used and the steady-state treatment of enzyme kinetics are strictly equivalent in their consequences. They are two ways of expressing the same basic phenomena. In that respect, when Goodman *et al.* [28] compute by two different methods error-frequencies, and then « prove », using a « Poisson model » that the results are related, they are merely checking the internal consistency of Mathematics.

The probabilistic approach is simpler when one is interested in comparing two rates rather than in knowing their absolute values. With the

steady-state treatment of the Michaelis equation one would obtain for instance :

$$V^S = \frac{[S]\,[E]\,k_2^S\,k_1^S\,(k_2^A + k_{-1}^A)}{[S]\,k_1^S\,(k_2^A + k_{-1}^A) + [A]\,k_1^A\,(k_2^S + k_{-1}^S) + (k_2^S + k_{-1}^S)\,(k_2^A + k_1^A)} \quad (27)$$

When making the ratio v^S/v^A, most of the terms cancel out and we are left with just the terms that we obtained directly by probability reasonnings. Probabilistic equations were developped by Knorre and Malyguine [20] for comparing the rates of two reactions occurring on a same enzyme (for instance the pyrophosphate exchange reaction versus the formation of the aminoacyl-adenylate on an aminoacyl-tRNA ligase). However, Knorre's group did not deal with discrimination problems.

A number of reports, often misleading, appeared recently linking accuracy to kinetics.

Thus, Yarus [31] claimed that « the very existence of parallel systems of tRNA and synthetase in the same cytoplasm suppresses misacylations which would otherwise occur ». Our treatment of the Michaelis scheme should make clear that under the condition of steady-state kinetics, there is no Yarus-effect to be expected.

Staying with the ligases, a point of logic can be made. When the incorrect aminoacyl-tRNA leaves the enzyme, it has a non-negligible probability (say 10 p. cent) of going to the ribosome. Therefore, the error has to be corrected *before* the tRNA leaves the enzyme. Having a proofreading function for that error on another ligase would bring limited improvements to accuracy. If there is any specificity to be expected in the hydrolytic function of the ligases, it should be preferentially directed towards the destruction of the erroneous products that are made by the enzyme itself. That point seems to have been overlooked by two independant groups of experimentalists [32, 33].

Bessman *et al.* [13] made experiments on the accuracy of DNA polymerization with the T4 enzymes, and checked their experimental results with a theoretical equation derived by Goodman *et al.* [28]. The authors did not realize that the « theoretical equation » was actually a mere *definition* linking their experimental quantities (their Equation (2) can be directly calculated from their set of Equations (1)). Thus, even if the authors had obtained their data by drawing lots, they would have been able to check identically their Equation (2). The source of their error can be traced back to a confusion in the theoretical paper [28]. When writing (p. 425) $R(B_j)$ = turn B_j/(inc B_j + turn B_j), the authors identified a probability of hydrolysis in the elementary step with an overall probability of hydrolysis in the complete process.

It is difficult to judge at the onset whether or not some enzymatic systems take advantage of the amplification possibilities that we have discussed. For one thing our analysis stresses the fact that accuracy may depend critically upon details to which no attention would be paid normally. For instance, any analysis of the hydrolytic activity in the aminoacyl-tRNA ligases should take into consideration the sequence of the events that lead to the departure of the tRNA from the enzyme. The reaction of degradation of ATP described in Equation (8) if observed would be considered as waste, or as due to *in vitro* artifacts.

One may ask why the cell is not working at the highest achievable level of accuracy. A possible answer is that efficiency and accuracy appear somehow antinomic. Consider the case of Equation (10). There is a good discrimination when k_{-1} is large compared to k_3 and k_{-2} and $k_2[S_2]$. Then accuracy may be controlled through the concentration of S_2. By lowering it one makes the reaction more accurate, but at the same time it appears less efficient : it takes more trials in order to have a successful incorporation.

All the reaction schemes that we have considered involve a last irreversible step. Taking into account the terminal reversibility makes the analysis far more complicated, but is a necessity for a valid evaluation of the energetic cost of accuracy. A partial solution to the problem may be provided by making the last step of the reaction reversible *and* including an additional irreversible step which represents the net consumption of the product by the next step of the metabolic net into which the considered reaction is imbedded.

Acknowledgments.

I am indebted to L. E. Orgel for the discussions from which this paper stemmed, and for going patiently through many of its earlier versions. The work was supported by the Edna McConnell Clark Foundation and by the C.N.R.S. (France).

RÉSUMÉ.

On a étudié par une approche probabiliste la relation entre la précision dont un système enzymatique est capable et le mécanisme réactionnel. Certains mé-

canismes dans lesquels une des étapes est retardée agissent comme des amplificateurs cinétiques des discriminations moléculaires. La relation entre notre schéma de réaction retardée (1) et le schéma de Hopfield (2) est discutée.

REFERENCES.

1. Ninio, J. (1975) in « *L'Evolution des Macromolécules Biologiques* » (C. Sadron, Ed.) Editions du C.N.R.S., Paris, in the press.
2. Hopfield, J. J. (1974) *Proc. Nat. Acad. Sci.*, USA, **71**, 4135-4139.
3. Koshland, D. E. (1958) *Proc. Nat. Acad. Sci.*, USA, **44**, 98-104.
4. Wolfenden, R. (1972) *Accounts Chem. Res.*, **5**, 10-18.
5. Speyer, J. F., Karam, J. D. & Lenny, A. B. (1966) *Cold Spring Harbor Symp. Quant. Biol.*, **31**, 693-697.
6. Drake, J. W. & Allen, E. F. (1968) *Cold Spring Harbor Symp. Quant. Biol.*, **33**, 339-344.
7. Freese, E. B. & Freese, E. (1967) *Proc. Nat. Acad. Sci.*, USA, **67**, 650-657.
8. de Vries, F. A. J., Swart-Idenburg, Ch. J. H. & de Waard, A. (1972) *Molec. Gen. Genet.*, **117**, 60-71.
9. Drake, J. W. (1973) *Genetics Supplement*, **73**, 45-64.
10. Hershfield, M. S. (1973) *J. Biol. Chem.*, **248**, 1417-1423.
11. Nossal, N. G. & Hershfield, M. S. (1973) in *DNA Synthesis in Vitro* (Wells, R. D. & Inman, R. B., eds.), pp. 47-62, University Park Press, Baltimore.
12. Muzyczka, N., Poland, R. L. & Bessman, M. J. (1972) *J. Biol. Chem.*, **247**, 7116-7122.
13. Bessman, M. J., Muzyczka, N., Goodman, M. F. & Schnaar, R. L. (1974) *J. Mol. Biol.*, **88**, 409-421.
14. Rosset, R. & Gorini, L. (1969) *J. Mol. Biol.*, **39**, 95-112.
15. Strigini, P. & Gorini, L. (1970) *J. Mol. Biol.*, **47**, 517-530.
16. Biswas, D. K. & Gorini, L. (1972) *J. Mol. Biol.*, **64**, 119-134.
17. Gorini, L. (1971) *Nature New Biol.*, **234**, 261-264.
18. Ninio, J. (1973) *Progr. Nucleic Acid Res. Mol. Biol.*, **13**, 301-337.
19. Ninio, J. (1974) *J. Mol. Biol.*, **84**, 297-313.
20. Knorre, D. G. & Malyguine, E. G. (1972) *Dokl. Akad. Nauk SSSR*, **207**, 1391-1393.
21. Brutlag, D. & Kornberg, A. (1972) *J. Biol. Chem.*, **247**, 241-248.
22. Hamilton, L., Mahler, I. & Grossman, L. (1974) *Biochemistry*, **13**, 1888-1896.
23. Eldred, E. W. & Schimmel, R. J. (1972) *J. Biol. Chem.*, **247**, 2961-2964.
24. Yarus, M. (1972) *Proc. Nat. Acad. Sci.*, USA, **69**, 1915-1919.
25. Bonnet, J., Giegé, R. & Ebel, J. P. (1972) *FEBS Letters*, **27**, 139-144.
26. Yarus, M. & Berg, P. (1969) *J. Mol. Biol.*, **42**, 171-189.
27. Eldred, E. W. & Schimmel, P. R. (1972) *Biochemistry*, **11**, 17-23.
28. Goodman, M. F., Gore, W. C., Muzyczka, N. & Bessman, M. J. (1974) *J. Mol. Biol.*, **88**, 423-435.
29. Hall, Z. W. & Lehman, I. R. (1968) *J. Mol. Biol.*, **36**, 321-333.
30. Howard, B. D. & Tessman, I. (1964) *J. Mol. Biol.*, **9**, 364-371.
31. Yarus, M. (1972) *Nature New Biol.*, **239**, 106-108.
32. Bonnet, J. & Ebel, J. P. (1974) *FEBS Letters*, **39**, 259-262.
33. Sourgoutchov, A., Blanquet, S., Fayat, G. & Waller, J. P. (1974) *Eur. J. Biochem.*, **46**, 431-438.