THE KINETICS OF MULTIPLE ENZYME INHIBITION*

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In the course of studies on the adaptive fermentation of galactose by yeast, the effects of a number of enzyme inhibitors on the adaptive process and the subsequent fermentation were investigated. The results of these experiments as they bear on the problem of adaptation have been discussed in a preliminary communication (1). However, one interesting point emerged in concentration curves for two of the inhibitors. Both sodium azide and sodium fluoride exhibited a stimulatory effect at sufficiently low concentrations.

This seemed particularly significant in view of the fact that Borei (2) had already reported such an effect for precisely the same compounds acting on the endogenous respiration of bakers' yeast. It therefore became of interest to see whether a general theoretical analysis of such an effect is possible. It proved feasible to develop at least a simple form of such an analysis for fluoride in terms of its known properties. An equally concrete model for azide has not been completed, but is now being studied.

Materials and Methods

A pure strain of Saccharomyces carlsbergensis (CLD-1A, obtained from Dr. S. Spiegelman) was grown in liquid culture containing peptone and 8 per cent glucose, together with yeast extract and the necessary salts. For experiments, 48 hour cultures were harvested, washed several times with m/15 KH₂PO₄ to remove adherent medium, and finally suspended in enough m/15 KH₂PO₄ to give about 20 mg. (wet weight) of yeast in 1 ml. of suspension. Aliquots of 1 ml. were distributed among conventional Warburg reaction vessels; enough galactose solution to make a final concentration of 4 per cent was placed in the side-arm of each vessel; and enough distilled water, inhibitor, or phosphate solution was added to the main chamber to bring the final volume of liquid to 2 ml. (Inhibitor solutions were adjusted to a pH of 4.5 before use.) In occasional experiments the concentration of yeast or of galactose was varied, but in any one experiment the conditions in all vessels were strictly comparable. After equilibration on the Warburg bath, the substrate was tipped into the main chamber, the time at which this occurred being designated as zero time. Oxygen consumption and CO₂ production were followed at 15 minute or sometimes at 10 minute intervals by the usual two-cup method (3). The excess of CO₂

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production over O₂ consumption in a given time period was taken as an index of fermentation and hence of adaptation rate. With this strain of yeast, adaptive fermentation first appears at 90 minutes, and increases in rate.

EXPERIMENTAL RESULTS

The effect of azide on the course of adaptation is summarized in Table I. The manometric procedures were as usual; the galactose concentration was 2 per cent. Azide, brought to pH 4.5, was added to the main cup about 15 minutes before the substrate. The excess of CO₂ over O₂ during the postadaptive period is used as the index of adaptation rate.

The effect of the higher azide concentrations, 10⁻⁴ and 10⁻⁸ M, is in line with expectations: oxygen consumption is strongly depressed, and adaptation, as measured by extra CO₂, is only 20 per cent of normal.

With the 10⁻⁸ M azide, however, despite an inhibition of oxygen uptake which is in line with the rest of the data, the rate of adaptation is stimulated by 75 per cent over the control value.

This effect is quite reproducible. In another experiment at 10⁻⁴ M, for instance, the O₂ consumption before and after adaptation was 53 and 78 per cent of the control, while the extra CO₂ was 139 per cent of the control value. Even more striking was an experiment in which the azide was tipped from a side-arm 45 minutes after the addition of galactose. In this case the O₂ rates were 60 and 65 per cent of the control value for the pre- and postadaptive periods, but the extra CO₂ was 265 per cent of the control figure.

An experiment on fluoride is summarized in Table II.

These results contrast with those for azide, in that much less significant inhibition of respiration is coupled with much more complete inhibition of adaptation. This would be reasonable if fluoride were acting chiefly on enolase, and preventing fermentation from being completed rather than preventing the formation of the adaptive enzyme.
The concentrations in the experiment of Table II were chosen so as to encompass the known range of maximal inhibition of such enzymes as enolase, adenylpyrophosphatase, and the phosphate transfer enzymes in cell-free systems. No stimulating effect was found, and an experiment at a lower concentration range was required to demonstrate that such an effect exists. This experiment is summarized in Table III.

### Table II

**Effect of NaF on Adaptation**

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Fluoride concentration</th>
<th>Control</th>
<th>5 × 10⁻⁴ M</th>
<th>10⁻⁴ M</th>
<th>2 × 10⁻⁴ M</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-90</td>
<td>O₂, microlitres</td>
<td>320</td>
<td>278</td>
<td>191</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>Per cent of control</td>
<td>—</td>
<td>87</td>
<td>60</td>
<td>22</td>
</tr>
<tr>
<td>90-150</td>
<td>O₂, microlitres</td>
<td>338</td>
<td>310</td>
<td>202</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>Per cent of control</td>
<td>—</td>
<td>92</td>
<td>60</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Excess CO₂, microlitres</td>
<td>111</td>
<td>19</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Per cent of control</td>
<td>—</td>
<td>17</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

### Table III

**Fluoride Stimulation of Adaptation**

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Fluoride concentration</th>
<th>Control</th>
<th>10⁻⁴ M</th>
<th>10⁻⁵ M</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-90</td>
<td>O₂, microlitres</td>
<td>690</td>
<td>688</td>
<td>708</td>
</tr>
<tr>
<td></td>
<td>Per cent of control</td>
<td>—</td>
<td>100</td>
<td>103</td>
</tr>
<tr>
<td>90-150</td>
<td>O₂, microlitres</td>
<td>740</td>
<td>743</td>
<td>738</td>
</tr>
<tr>
<td></td>
<td>Per cent of control</td>
<td>—</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Excess CO₂, microlitres</td>
<td>164</td>
<td>191</td>
<td>205</td>
</tr>
<tr>
<td></td>
<td>Per cent of control</td>
<td>—</td>
<td>117</td>
<td>125</td>
</tr>
</tbody>
</table>

The stimulating effect on excess CO₂ is well outside the experimental error. Nevertheless, it is small compared with that of azide. It is interesting that it occurs at a much lower concentration than with azide and at a concentration at which oxidation is completely unaffected. These findings contrast with those of Borei (2), who found the maximum stimulation of endogenous respiration by NaF at about 0.02 M.

**Theory**

Spiegelman and Reiner (4) have studied mathematically a number of reaction systems which possess the property that the over-all reaction rate, con-
sidered as a function of concentration of some particular substance, rises to a maximum and subsequently diminishes, so that the substance in question has a stimulatory effect at low and an inhibitory effect at high concentrations—a property which many narcotics and poisons have been shown to possess.

The systems previously analyzed, however, have been distinguished by the fact that the critical substance was assumed to act at only one step or level of the system. The double property of stimulating and inhibiting resulted from such things as geometrical conditions of constraint upon the reactants. For example, if two substrates react together to form a product, and if they must lie side by side on the surface of an enzyme in order to react, each of the substrates stimulates the reaction rate with increasing concentration up to a certain point, and then becomes inhibitory with further increase.

It is of some interest to consider the situation in which a substance acts at more than one step of the reaction system, and acquires the double property of stimulating and inhibiting in this way.

The reason for analyzing the effects of fluoride in terms of a multiple inhibitory action is that it is already known to act upon several different steps of the Meyerhof scheme, such as enolase, adenosinetriphosphatase (ATPase), and the phosphate transfer from phosphopyruvate to adenosinediphosphate or adenylic acid. Moreover, we can readily visualize in a purely qualitative way how the inhibition of ATPase, for instance, might be stimulating. If phosphorylation of hexose by ATP were the limiting step of a reaction chain, not because the enzyme is limiting but because the supply of ATP is limited, then ATPase would limit the rate, while an inhibitor of ATPase would consequently have the effect of increasing the ATP supply and so increase the rate. As the inhibitor concentration is increased, other reaction steps would be affected, so that eventually inhibition would manifest itself.

We consider now a somewhat simplified reaction system which has the above properties.

\[
\begin{align*}
A & \xrightarrow{1} B \xleftarrow{2} C \rightarrow X \\
& \downarrow D
\end{align*}
\]

In the above diagram, \(A\) represents a substrate, whose concentration is maintained constant, either by diffusion or by making the initial amount so large that the concentration does not vary appreciably during the time of an experiment. \(B\) is a phosphorylated intermediate, and \(C\) is the substance whose rate we are measuring. By introducing a last step by means of which \(C\) is removed, it is possible to consider the reaction system in a steady state, and so to eliminate a number of inessential mathematical complications. Reaction 3 is taken to represent the effect of ATPase, by removing the phosphorylated
intermediate B along a "blind alley." This again is not a perfectly accurate representation, but is also used for the sake of simplicity (if all reactions can be written as if they were monomolecular, the complexity of the algebra is greatly reduced, and the essential features of the system are retained). Reaction 2 may be thought of as representing the enolase step or the dephosphorylation of PPA. In disregarding a number of intermediate steps, we are implying that they are not limiting under the conditions of the experiment.

The kinetic equations of the system now are:

\[
\frac{dB}{dt} = k_1A - k_1'B + k_1'C - k_2B - k_3B
\]

\[
\frac{dC}{dt} = k_2B - k_2'C - hC
\]

The capital letters now represent concentrations; we omit the usual brackets around them to keep the notation simple, since no misunderstanding can arise.

The condition for a steady state is that the time derivatives shall vanish. This condition is equivalent to setting the right-hand sides of the equations equal to zero; and this yields two linear simultaneous equations for determining the steady state values of B and C, which we shall denote by \(B^*\) and \(C^*\). We now write down the solutions, omitting the elementary manipulations which are required to obtain them:

\[
B^* = \frac{k_1Ak_1'/(k_1' + k_1)(k_1' + h) + hh_1}{k_2B - k_2'C - hC}
\]

\[
C^* = \frac{k_2Ak_2'/(k_1' + k_2)(k_1' + h) + hh_1}{k_2B - k_2'C - hC}
\]

The rate at which C is produced by reaction 2, which is what we are interested in studying, is \(k_2B^* - k_2'C^*\). But from the \(dC/dt\) equation above we can see that, in the steady state, this expression is equal to \(hC^*\) (the rate of production equals the rate of removal; e.g., by neutralization or diffusion). We can therefore write:

\[
R = hC^* = \frac{hkBk_1A}{(k_1' + k_1)(k_1' + h) + hh_1}
\]

The condition that reactions 2 and 3 are affected by fluoride can be written:

\[
k_1 = k_1(1 - aF) \quad k_1' = k_1'(1 - aF) \quad k_2 = k_2(1 - bF)
\]

where F represents the concentration of fluoride. Treating the effect of fluoride on each reaction rate constant as a linear function of F is of course an approximation; we have no way of knowing the exact relation, and choose to begin with the simplest expression possible. The constants a and b may be termed the sensitivities of the respective reactions to fluoride; for, the larger they are, the smaller will be the concentration of fluoride which is required to produce a given degree of inhibition. If we substitute these expressions directly into the rate R given above, we will have it expressed as a function of the fluoride concentration.
To determine whether $R$, considered as a function of $F$, has a maximum, we need only obtain the derivative $dB/dF$, set it equal to zero, and solve for $F$ in the resulting equation. If the solution is real, then it is either a maximum or a minimum, and we can proceed to determine which.

Again omitting some simple manipulations, we write down the equation which results from setting $dR/dF$ equal to zero:

$$(aF)^2 - 2aF + 1 + k/b_k's - ka(1 + k'/k_{a_0})/b b_k's = 0.$$ 

This is a quadratic equation, which has two solutions:

$$F = \frac{1 \pm \sqrt{(h/k_{a_0})(a(1 + k'/k_{a_0})/b - 1)}}{a}.$$ 

The condition for the solution to be real is that the radicand be positive or zero, but not negative:

$$a(1 + k'/k_{a_0})/b \geq 1.$$ 

This condition can be interpreted to mean that the sensitivity of e.g., enolase $(a)$ must not be too small, or that of ATPase $(b)$ too large; otherwise only a stimulating effect will be found without any eventual inhibition.

It is evident that we must discard the solution with the positive sign of the radical; for this would make $aF$ always greater than 1, which would make the reaction rate negative—a physically meaningless result.

We wish to insure that the remaining solution, with the negative sign of the radical, shall give positive as well as real values of $F$, since a negative concentration also is meaningless, and a maximum at $F = 0$ would of course mean that in reality we observe no maximum. The condition will obviously be fulfilled if the expression under the radical sign is smaller than 1:

$$a(1 + k'/k_{a_0})/b < 1 + k_{a_0}'/k.$$ 

This and the inequality just preceding prescribe an interval within which the ratio $a/b$ must lie if we are to obtain a fluoride concentration curve with a maximum.

The fact that a maximum, rather than a minimum, is present under these conditions is easily verified, either by using the second derivative $(d^2R/dF^2)$ as is usual in calculus, or even more simply by finding the initial value of the first derivative $dR/dF$—its value for $F = 0$. The condition that this quantity shall be positive turns out to be identical with the inequality just stated which guarantees a positive extremal value for $F$. But if we have a positive initial slope, this means that the $F$ concentration curve begins to rise; this means that for low values of $F$ we are getting stimulation, so that the extremal value must be a maximum rather than a minimum.

We can see that the solution just found makes sense by considering the expression for the value of $F$ at the maximum, which we have previously written...
down. This depends on \( c \), and on the ratio \( a/b \). For the moment, consider \( a \) to be fixed, and vary \( b \) so as to vary \( a/b \). The smaller \( a/b \), i.e., the larger \( b \), the larger is \( F \). That is, for a given fluoride sensitivity of enolase, the larger is the sensitivity of ATPase, the higher will be the fluoride concentration required before inhibition is observed—a result which agrees well with our intuitive qualitative analysis of the situation.

In carrying out this mathematical analysis, it has been implicitly assumed that the system is not limited by the concentration of phosphate acceptor, but only by that of phosphate donor. Actually, there may arise situations in which the concentration of ADP decreases so much as to be limiting; such a case has been considered by Meyerhof (5), who attributes the slow rate of HDP fermentation as against glucose fermentation to inactivity of ATPase.

The exact analysis of this case is somewhat complex, and has not yet been satisfactorily completed. We may, however, get some idea of how it would behave from a study of the model system which we have set up in the foregoing. Let us for the moment ignore the problem of enolase, and interpret reaction 2 as the phosphate transfer from PGA or PPA to ADP. Inhibition of ATPase, by cutting down the supply of ADP, would also inhibit this reaction. To a first approximation, we might argue that \( a \) is equal to \( b \) in this case, since both effects of fluoride are actually on one reaction—the ATPase. If we put \( a \) equal to \( b \) in all the expressions which we have derived above, the conditions for a maximum in the fluoride concentration curve depend only on the various rate constants of the (uninhibited) system. The resulting pair of conditions can be expressed as follows:

\[
\frac{k}{k_1} < \frac{k_2}{k_1} \leq 1.
\]

It is very probable, with the approximations introduced, that the above inequality is far from exact for the system which it is supposed to represent. We have included it largely to give an example of the sort of conditions under which an inhibitor acting on a single reaction step might stimulate at low concentrations; more examples have been considered in the paper by Spiegelman and Reiner (4).

**SUMMARY**

The adaptive fermentation of galactose by yeast is inhibited by fluoride and azide. At sufficiently low concentrations, however, it is stimulated.

From a mathematical analysis, using the known fact that fluoride inhibits the enzymes enolase and adenosinetriphosphatase, it is possible to infer the existence of such stimulation. Conditions for this effect are derived which relate the sensitivities of the enzymes to the poison with the rate constants of the various reactions in the fermentation chain.
BIBLIOGRAPHY