Jeppsson, J. O., and Sjöquist, J. (1967), *Anal. Biochem. 18,* 264.
Matsuo, H., Fujimoto, Y., and Tatsuno, T. (1966), *Biochem. Biophys. Res. Commun. 22,* 69.
Moore, S. (1963), *J. Biol. Chem. 238,* 235.
Randerath, K. (1963), Thin-layer Chromatography (translated by Libman, D. D.), Weinheim/Bergstr., Germany, Verlag Chemie, p 93.
Smithies, O., Gibson, E., Fanning, M., Goodfliesh, R. M., Gulman, J. G., and Ballantyne, D. L. (1971), *Biochemistry 10,* 4912.
Stanier, R. Y., and Ingraham, J. L. (1954), *J. Biol. Chem. 210,* 799.
Stark, G. R., and Smyth, D. G. (1963), *J. Biol. Chem. 238,* 214.
Vesterberg, O., and Svensson, H. (1966), *Acta Chem. Scand. 20,* 820.
Waller, J. P. (1963), *J. Mol. Biol. 7,* 483.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.

# Lysine-Sensitive Aspartokinase of *Escherichia coli* K12. Synergy and Autosynergy in an Allosteric V System†

Jean-Pierre Mazat* and Jean-Claude Patte‡

ABSTRACT: The interactions of the lysine-sensitive aspartokinase of *E. coli* K12 with lysine and leucine, as evidenced in the inhibition and binding curves, are well explained by the equations of an allosteric V model. Mathematical treatments of such a model lead to new linearized plots. These representations are applied to our experimental results and allow the direct determination of some parameters of the model (equilibrium constant $L'$ and leucine dissociation constants). The other parameters are obtained by an optimization method. The theoretical curves drawn according to this model account well for the synergistic inhibition between lysine and leucine and for the role of the two nonequivalent lysine binding sites ("autosynergy").

The lysine-sensitive aspartokinase of *Escherichia coli* K12 (aspartokinase III)[1] is one of the three enzymes catalyzing the phosphorylation of the β-carboxyl of aspartate in this bacteria; L-lysine is the feedback inhibitor of this activity. Lysine inhibition is cooperative (Stadtman et al., 1961; Patte et al., 1967).

It has been shown that the protein is a dimer of mol wt 105 000 composed of two identical subunits (Richaud et al., 1973). There are two nonequivalent binding sites for lysine per subunit, i.e., four lysine sites/enzyme molecule (Richaud et al., 1974).[2] The binding of lysine is cooperative (Richaud et al., 1974).

Nonspecific amino acids such as leucine, isoleucine, etc., also substantially inhibit the enzymatic activity; a synergistic inhibition is observed between lysine and these amino acids (Patte et al. 1965). The leucine binding site(s) appears different from the two lysine sites (Richaud et al., 1974) (as their number is not experimentally measurable, the simplest assumption, that there exists only one leucine binding site per subunit, i.e., two leucine sites/enzyme molecule, will be used in the following).

Monod et al. (1965) have proposed aspartokinase III as an allosteric V system for the following reasons: no cooperative effect of substrates is observed, and lysine and leucine are strictly noncompetitive inhibitors toward aspartate (Stadtman et al., 1961; Truffa-Bachi et al., 1966; Wampler and Westhead, 1968).

This hypothesis was further strengthened by recent results of our laboratory: the sigmoidicity of the lysine binding curve disappears when binding is performed in the presence of leucine, a synergistic inhibitor (Richaud et al., 1974). This could be explained by a shift of an allosteric equilibrium (Monod et al., 1965). Moreover, physical techniques suggest the existence of different forms for the protein, depending on the presence of the inhibitory ligands (Richaud et al., 1974).

In this paper we will show that an allosteric V model explains quantitatively both kinetic and binding results obtained with the inhibitory ligands, and particularly the synergistic inhibition. The parameters of the model (dissociation constants for the inhibitors and equilibrium constants) will be estimated.

## Materials and Methods

*Strains Used and Enzyme Purification.* Aspartokinase III was purified from *E. coli* K12 strain D06 according to the previously published procedure (Lafuma et al., 1970; Richaud et al., 1973). For kinetic measurements, the partially pure fraction, obtained after the DEAE-Sephadex step, was used (sp act. 5550 nmol $min^{-1}$ $mg^{-1}$).

† From the Laboratoire de Biochimie B.B.C., Université de Bordeaux II, 33405 Talence, France (J.-P.M.) and Institut de Microbiologie, Université de Paris-Sud, 91405 Orsay, France (J.-C. P.). *Received February 3, 1976.* Financial support came from the Centre National de la Recherche Scientifique, the Delegation Générale à la Recherche Scientifique et Technique and the Fondation pour la Recherche Médicale Française. This work is part of the Thesis to be submitted by J. P. Mazat, in partial fulfillment of the requirements for the Doctorat-ès-Sciences. A preliminary report of this work has been given at the 9th FEBS Meeting (Budapest, 1974).

[1] Abbreviations used are: AK III or aspartokinase III, L-lysine-sensitive aspartokinase of *E. coli* K12 (EC 2.7.2.4); DEAE, diethylaminoethyl.

[2] Recently Funkhouser et al. (1974) have obtained, in the case of aspartokinase III of *E. coli* B, one binding site only for lysine per enzyme molecule; however, it is not known whether the subunits of this enzyme are identical. Also, saturation of binding is not reached in their experiments.

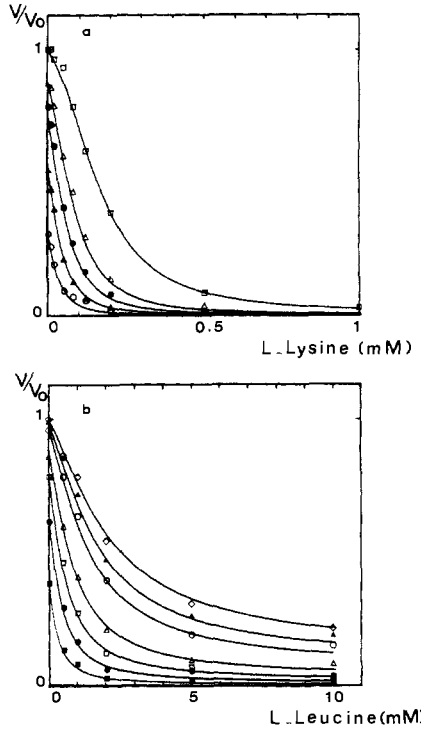


FIGURE 1: Inhibition curves of AK III in the simultaneous presence of lysine and leucine. (a) Inhibition by lysine at various leucine concentrations: (□) 0; (△) 0.5 mM; (●) 1 mM; (▲) 2 mM; (○) 5 mM. (b) Inhibition by leucine at various lysine concentrations: (◇) 0; (▲) 0.01 mM; (○) 0.02 mM; (△) 0.05 mM; (□) 0.08 mM; (●) 0.12 mM; (■) 0.2 mM. The points show the experimental values; the curves are drawn according to eq 1 with: $L' = 0.04$; $K_R^{Leu} = 6$ mM; $K_T^{Leu} = 0.4$ mM; $K_R^{1,Lys} = 0.8$ mM; $K_R^{2,Lys} = 1.6$ mM; $K_T^{1,Lys} = 0.07$ mM; $K_T^{2,Lys} = 0.15$ mM.

*Materials.* L-Aspartate, ATP (disodium salt), L-lysine, and L-leucine were of analytical grade, purchased from Sigma Chemical Co.

*Assay Procedure.* Aspartokinase activity was measured by hydroxamate ferric chloride assay (Stadtman et al., 1961, modified according to Patte et al., 1966).

*Computing Techniques.* For drawing the theoretical curves, a Hewlett Packard 9821A calculator and 9822A Plotter were used.

The optimization of parameters of eq 1 and 11 was made according to a simplex method (see, for example, Swann, 1969). All programs were written in Fortran IV and carried out with an IBM 1130 computer. The criterion of optimization was the minimization of the sum $\Sigma_i(o_i - c_i)^2$ where $o_i$ is the experimental value and $c_i$ the corresponding calculated value. Constraints were introduced in the program: positivity of parameters; $K_R^{1,Lys} < K_R^{2,Lys}$ and $K_T^{1,Lys} < K_T^{2,Lys}$ to avoid convergence toward symmetric sets of parameters. With these conditions the only good fitting of the experimental results (according to the criterion) is obtained when the different parameters are in the range given in Table I.

## Results

### (a) *Inhibition Curves of AK III in the Simultaneous Presence of Lysine and Leucine*

The inhibition of AK III by lysine and leucine has been determined at several concentrations of each amino acid (both substrates being present at saturation). Results are given in Figure 1: in Figure 1a, the rate is given as a function of the lysine concentration, at various concentrations of leucine; in Figure 1b, the reverse representation is used (i.e., the rate as a function of the leucine concentration at various concentrations of lysine). Points represent experimental values (it must be noted that, for values lower than 0.1, a great imprecision is observed due to the assay method). The curves are theoretical ones, drawn using kinetic parameters determined as explained below.

These data have been treated according to the equations derived from the model of Monod et al. (1965) for the V class of allosteric enzymes. As defined by these authors, this class of enzymes is characterized by the fact that a substrate has the same affinity for the two states R and T of the enzymatic protein, the regulation of the enzyme activity by the binding of a ligand being obtained through modifications of the allosteric equilibrium between the R and T forms.

In the case of AK III, the following features must be taken into account: (1) As a total inhibition of enzyme activity is observed (Figure 1), the T form of the enzyme must be inactive. (2) By protomer two nonequivalent binding sites exist for lysine (which dissociation constants for the R and T forms will be called, respectively, $K_R^1$, $K_T^1$, $K_R^2$, and $K_T^2$). Leucine is also an inhibitory ligand (with dissociation constants $K_R$ and $K_T$). (3) As the active protein is in a dimeric state, the modification of the allosteric equilibrium by the binding of a ligand to one site will be obtained through terms of the following (defined as *allosterian:* $A_{ligand}$ for the sake of simplicity):

$$(1 + (Leu)/K_T)^2/(1 + (Leu)/K_R)^2 = A_{Leu}$$

$$(1 + (Lys)/K_T^1)^2/(1 + (Lys)/K_R^1)^2 = A_{Lys}^1$$

$$(1 + (Lys)/K_T^2)^2/(1 + (Lys)/K_R^2)^2 = A_{Lys}^2$$

Then the basic equation of Monod et al. (1965) for velocity, at saturation for all substrates, becomes, extended to the binding of several inhibitors:

$$\frac{V}{V_0} = \frac{1 + L'}{1 + L'A_{Leu}A_{Lys}^1A_{Lys}^2} \quad (1)$$

where the equilibrium constant $L'$ and the rate $V_0$ are the parameters in the absence of lysine and leucine.

In the presence of lysine alone:

$$\frac{V_{Lys}}{V_0} = \frac{1 + L'}{1 + L'A_{Lys}^1A_{Lys}^2} \quad (2)$$

In the presence of leucine alone:

$$\frac{V_{Leu}}{V_0} = \frac{1 + L'}{1 + L'A_{Leu}} \quad (3)$$

### (b) *Hill Plots*

The kinetic Hill representation for one inhibitory ligand, log $[(V_m - V)/V]$ vs. log (ligand), may be extended in our case, as different Hill plots may be obtained for one ligand in the presence of different concentrations of the other ligand.

The Hill plot for lysine at a given concentration of leucine will be: log $[(V_{Leu} - V)/V]$ vs. log (Lys), $V_{Leu}$ being the rate in the presence of this concentration of leucine alone. From eq 1 and 3:

$$\log \frac{V_{Leu} - V}{V} = \log \frac{L'A_{Leu}}{1 + L'A_{Leu}} + \log (A_{Lys}^1A_{Lys}^2 - 1) \quad (4)$$

Using different leucine concentrations (that lead to different $V_{Leu}$ values), it appears from eq 4 that the curves obtained will remain *parallel.* The reverse is true (Hill plots of leucine at

various concentrations of lysine). This parallelism, not observed in the case of an allosteric K system, may be considered as a salient feature of the allosteric V systems.

This has been tested on our experimental data. Figure 2a represents Hill plots as a function of the lysine concentration at various leucine concentrations; in Figure 2b the reverse situation is presented, plots being made as a function of the leucine concentration at various lysine concentrations. The points represent experimental values, the curves are theoretical ones, drawn using kinetic parameters determined as explained below. It can be seen that the results are in agreement with the expected parallelism, though at high concentrations of both inhibitors some points are scattered due to the imprecision in the experimental values, as already pointed out (this imprecision being magnified with the representation used).

These results thus favor the hypothesis that AK III belongs to the V class of allosteric enzymes.

### (c) *Determination of L'*

Linearization of eq 1 can be obtained, graphically leading to determination of some parameters of the system.

*Theoretical Equations.* The values for $A_{Lys}^1 \times A_{Lys}^2$ and $A_{Leu}$ may be derived from eq 2 and 3 and introduced in eq 1:

$$\frac{V}{V_0} = \frac{L'V_{Lys}V_{Leu}}{(V_0 - V_{Lys})(V_0 - V_{Leu}) + L'V_0^2} \quad (5)$$

where $V_{Lys}$ is the rate with lysine alone, $V_{Leu}$ the rate with leucine alone, $V$ being the rate with both inhibitors and $V_0$ the rate in the absence of both. Thus eq 5 may be written:

$$\frac{1}{V} = \frac{1}{V_{Lys}}\frac{1}{L'}\left[(1 + L')\frac{V_0}{V_{Leu}} - 1\right] + \frac{1}{L'}\left(\frac{1}{V_0} - \frac{1}{V_{Leu}}\right) \quad (6)$$

From this equation, it appears that if, for example, the concentration of leucine remains constant, i.e., $V_{Leu}$ having a fixed value, the plot of $1/V$ vs. $1/V_{Lys}$ at different lysine concentrations gives a straight line. Its intercept $I_{Leu}$ with the ordinates is equal to:

$$I_{Leu} = \frac{1}{L'}\left(\frac{1}{V_0} - \frac{1}{V_{Leu}}\right) \quad (7)$$

Different values of $I_{Leu}$ are obtained for different leucine concentrations. Thus, plotting $I_{Leu}$ vs. $1/V_{Leu}$, one again obtains a straight line. Its intercept on the ordinates is $1/L'V_0$, allowing a determination of the value of $L'$.

It should be noticed that all straight lines ($1/V$ vs. $1/V_{Lys}$ and $I_{Leu}$ vs. $1/V_{Leu}$) intercept at the same point, $\Omega$, the coordinates of which are: $X_\Omega = Y_\Omega = 1/[(1 + L')V_0]$.

The analogous plots, $1/V$ vs. $1/V_{Leu}$ for a fixed value of $V_{Lys}$, and $I_{Lys}$ vs. $1/V_{Lys}$, can be made; the intercept of the straight lines obtained is the same as above.

The secondary graphs of the two representations obey the same equation, eq 7, and must give the same straight lines.

One must point out that this simple representation does not involve the knowledge of either the number of ligand binding sites or of the dissociation constants and thus may be of general application.

*Experimental Results.* The data of Figure 1 have been plotted according to eq 6. In Figure 3a, $1/V$ is plotted vs. $1/V_{Lys}$; in Figure 3b, $1/V$ vs. $1/V_{Leu}$. The thick straight lines correspond to the plot according to eq 7.

It can be seen in Figure 3a and 3b that experimental points are fitted well by straight lines, all of which intercept at the same point $\Omega$ that corresponds to $V = V_M^R$ where $V_M^R$ is the maximal rate of the R form (this point is an asymptotic one corresponding to all the enzyme being in the R form).

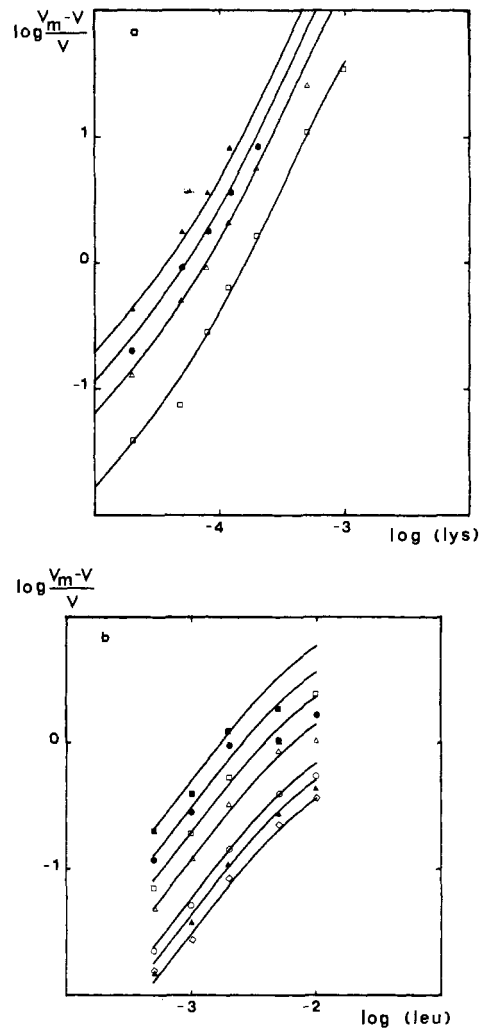


FIGURE 2: Hill representation of points of figure 1. (a) Inhibition by lysine at various leucine concentrations: (□) 0; (△) 0.5 mM; (●) 1 mM; (▲) 2 mM. (b) Inhibition by leucine at various lysine concentrations. (◇) 0; (▲) 0.01 mM; (○) 0.02 mM; (△) 0.05 mM; (□) 0.08 mM; (●) 0.12 mM (■) 0.2 mM. In the calculation of log $[(V_m - V)/V]$, $V$ is the experimental value, but $V_m$ is the theoretical value calculated from eq 1 using the constants quoted in the legend of Figure 1. The curves are theoretical ones.

The intercept of the secondary plots (eq 7) with the ordinates is $1/L'V_0$. As expected, the two secondary plots ($I_{Leu}$ vs. $1/V_{Leu}$ and $I_{Lys}$ vs. $1/V_{Lys}$) are not significantly different. Thus, for more precise determination of $L'$, the least-squares straight line has been obtained using points from both curves (data not shown), leading to a value of $L'$ equal to: $L' = 0.04 \pm 0.01$.

### (d) *Determination of the Dissociation Constants*

*Theoretical Equations.* From eq 2, we get: $L'A_{Lys}^1A_{Lys}^2 = [(1 + L')V_0 - V_{Lys}]/V_{Lys}$. Then, if the lysine concentration is fixed, we obtain from eq 1:

$$A_{Leu} = \frac{(1 + (Leu)/K_T)^2}{(1 + (Leu)/K_R)^2} = \frac{V_0(1 + L') - V}{V} \times \frac{V_{Lys}}{(1 + L')V_0 - V_{Lys}} \quad (8)$$

In the same manner, from eq 3 and 1, we may obtain:

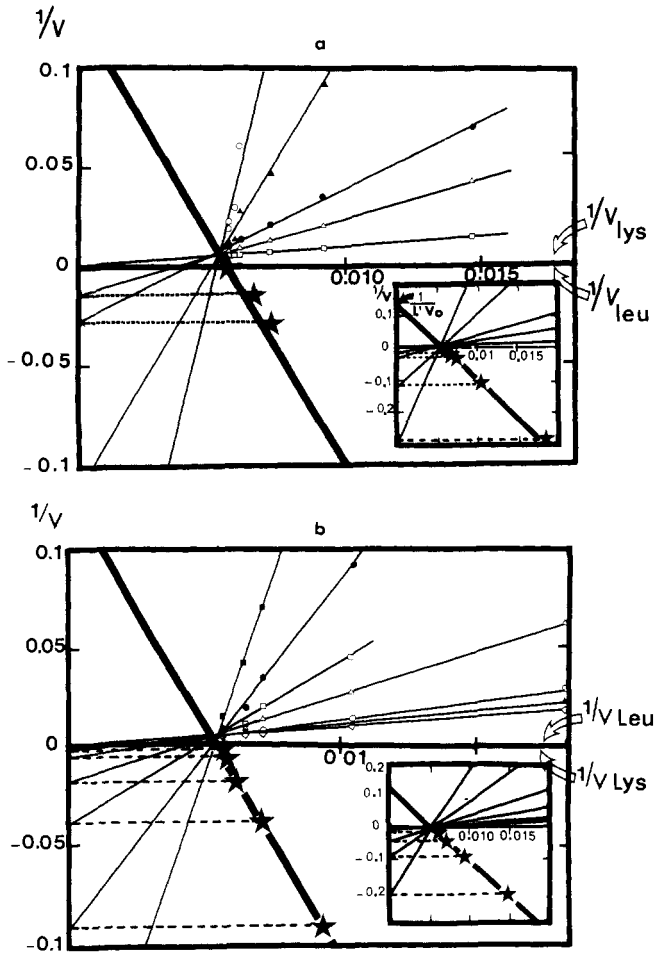


FIGURE 3: Linearization of curves of Figure 1 according to eq 6 and 7. (a) $1/V$ plotted vs. $1/V_{Lys}$ (variation of lysine at various leucine concentrations). Thick line represents the secondary graph according to eq 7. For this latter graph, $1/V_{Leu}$ is plotted on the abscissa with the same scale as one for $1/V_{Lys}$, so as to evidence the common intercept point, Ω. (b) $1/V$ plotted vs. $1/V_{Leu}$ (variation of leucine at various lysine concentrations). Thick line represents the secondary graph as in figure 3 a. The inserts give all the straight lines for each representation and the linearization of their intercept on the ordinate according to eq 7. The straight lines are given by least-squares method.

$$A_{Lys}{}^{1} \times A_{Lys}{}^{2} = \frac{(1 + (Lys)/K_T{}^{1})^2}{(1 + (Lys)/K_R{}^{1})^2} \frac{(1 + (Lys)/K_T{}^{2})^2}{(1 + (Lys)/K_R{}^{2})^2} = \frac{V_0(1 + L') - V}{V} \times \frac{V_{Leu}}{(1 + L')V_0 - V_{Leu}} \quad (9)$$

*Leucine Dissociation Constants.* From eq 8 we can extract:

$$\frac{1}{\sqrt{A_{Leu}} - 1} = \frac{1}{(Leu)} \times \frac{K_R K_T}{K_R - K_T} + \frac{K_T}{K_R - K_T} \quad (10)$$

Plotting $1/(\sqrt{A_{Leu}} - 1)$ vs. $1/(Leu)$, we may draw a straight line which intercepts the abscissa at $-1/K_R$ and the ordinates at $K_T/(K_R - K_T)$. Such a plot is presented in Figure 4.

The symmetric plot $1/[(1/\sqrt{A_{Leu}}) - 1]$ vs. $1/(Leu)$ similarly leads to a straight line, giving a determination of $1/K_T$ (data not shown). In both cases experimental points fit well straight lines. The obtained constants are: $K_R{}^{Leu} = 6$ mM and $K_T{}^{Leu} = 0.4$ mM.

*Lysine Dissociation Constants.* In eq 9 the four lysine dissociation constants are involved; no linearization is possible in this case. In order to get an approximate value for these four constants, an optimization method was applied to the results

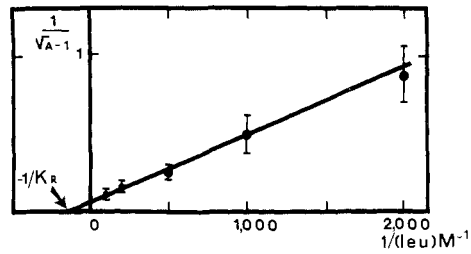


FIGURE 4: Linearization of inhibition curves by leucine (Figure 1b) according to eq 10. Points represent mean values for various lysine concentrations. All the points are in the range delimited by the segment (●), and are used in the determination of the straight line by the least-squares method.

of Figure 1, as described in Materials and Methods, according to eq 1. The same optimization method was also used to treat the experimental values obtained by lysine binding to the protein in the presence or in the absence of leucine (Richaud et al., 1974), according to the binding equation:

$$\bar{Y} = \frac{P(R) + L'P(T)}{Q(R) + L'Q(T)} \quad (11)$$

where

$$P(R) = \frac{(Lys)}{K_R{}^{1}}\left(1 + \frac{(Lys)}{K_R{}^{1}}\right)\left(1 + \frac{(Lys)}{K_R{}^{2}}\right)^2 + \frac{(Lys)}{K_R{}^{2}}\left(1 + \frac{(Lys)}{K_R{}^{1}}\right)^2\left(1 + \frac{(Lys)}{K_R{}^{2}}\right)$$

and

$$Q(R) = 2\left(1 + \frac{(Lys)}{K_R{}^{1}}\right)^2\left(1 + \frac{(Lys)}{K_R{}^{2}}\right)^2$$

$P(T)$ and $Q(T)$ are symmetric expressions involving the constants $K_T{}^{1}$ and $K_T{}^{2}$ instead of $K_R{}^{1}$ and $K_R{}^{2}$, respectively.

Such a method gives not only values for the lysine dissociation constants but also, for both velocity and binding experiments, additional and separate determinations of $L'$ and, for velocity experiment only, of $K_R$ and $K_T$ for leucine. However, such a method gives only the two lysine constants for the R form and the two lysine constants for the T form, without ascribing each value for one type of site (1 or 2). We have thus arbitrarily chosen to relate the low dissociation constants for one site, and the high dissociation constants for the other site. It must be also pointed out that, in some cases and especially for $K_R{}^{1}$ and $K_R{}^{2}$ values, the optimization method leads to a broad range of values consistent with the experimental data.

All these results are given in Table I in which previously published values are also reported (Richaud et al., 1974) for some parameters: $K_T{}^{1}$ and $K_T{}^{2}$ for lysine obtained by Scatchard plots of lysine binding and a $K_d$ apparent for leucine derived from differential spectra experiments.

Using the parameters determined by the optimization method and the equation described above, theoretical curves may be obtained for enzyme inhibiton by lysine and leucine, Hill plots, and lysine binding in the presence and in the absence of leucine. These curves are given in Figures 1, 2, and 5, respectively. It can be seen that in all cases a good agreement is observed between experimental points and theoretical curves.

## Discussion

The V class of allosteric enzymes, as defined by Monod et al. (1965), has been studied much less than enzymes belonging

TABLE I: Summary of the Results of Constant Determination by the Various Methods Quoted in the First Column.

| Mode of Determination | $L'$ | Leucine | | Lysine | | | |
|---|---|---|---|---|---|---|---|
| | | $K_R$ (mM) | $K_T$ (mM) | $K_R^1$ (mM) | $K_T^1$ (mM) | $K_R^2$ (mM) | $K_T^2$ (mM) |
| Plots of Figure 3 (according to eq 6 and 7), and plot of Figure 4 (according to eq 10)[a] | 0.04 ± 0.01 | 6 ± 1 | 0.4 ± 0.05 | | | | |
| Optimization method of kinetic data[b] | 0.04-0.05 | 5-7 | 0.3-0.5 | 0.6-1.2 | 0.07-0.1 | >1.6 | 0.1-0.2 |
| Optimization method of binding data[b] | 0.02-0.07 | | | 0.1-1 | 0.006-0.008 | >1 | 0.1-0.2 |
| Experimental results[c] (binding data) | | 1.15 | | 0.008 | | 0.1 | |

[a] The plots are straight lines given by the least-squares method. [b] Ranges of optimized values are given (see Materials and Methods). [c] Richaud et al. (1974).

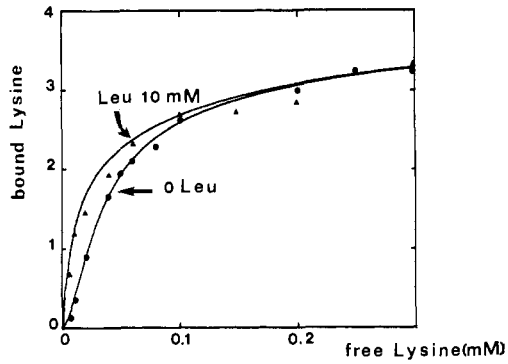


FIGURE 5: Binding curves according to eq 11. The points show experimental values (Richaud et al. 1974). The curves are drawn according to eq 11 with $L' = 0.04$ for leucine = 0 and $L' = 0.7$ for leucine 10 mM (the determination of $K_R^{Leu}$ and $K_T^{Leu}$ is not possible with only one leucine concentration); $K_R^{1,Lys} = 0.8$ mM; $K_R^{2,Lys} = 1$ mM; $K_T^{1,Lys} = 6$ μM; $K_T^{2,Lys} = 0.15$ mM. (●) leucine 0; (▲) leucine 10 mM.

to the K system. In fact, it appears that there are few examples of V enzymes for which a good knowledge of the protein structure and of the ligand binding sites exists.

During our study of the lysine-sensitive aspartokinase of *E. coli*, a possible example of a V enzyme, it appeared possible to get, after mathematical treatments of basic equations of Monod et al. (1965), simple representations allowing the determination of some kinetic parameters.

As described above, one linear representation, according to eq 6 and 7, leads to the determination of the equilibrium constant $L'$ between the T and R forms, and another representation according to eq 10 leads to the determination of the dissociation constants for one inhibitory ligand (if only one binding site per protomer exists for this ligand). These equations may be generally applied to any V system in which the T form is totally inactive, which is the most frequently encountered. It must be emphasized that the determination of $L'$ using the representation from eq 7 does not involve the knowledge of the number of inhibitory binding sites nor the dissociation constants for the ligands.

We have thus used these equations and these representations to test whether our experimental data obtained with aspartokinase III will fit with the theoretical statements. A good agreement was observed in the following cases: (a) Hill plots drawn at various concentrations of one inhibitory ligand as a function of the other ligand are parallel (Figure 2). (b) A linearization is observed when using eq 6, 7, and 10 (Figures 3 and 4). (c) The theoretical curves drawn, using parameters obtained by an optimization method according to the basic equations of a V system, are in good agreement with the experimental points, for inhibition curves as well as for binding experiments (Figures 1, 2, and 5). (d) When comparing the different values obtained separately for the different parameters (Table I), it appears that a good agreement is observed: the values for $L'$ are identical and the different dissociation constants are of the same order of magnitude (taking into account the broad range of values of $K_R^1$ and $K_R^2$ obtained by optimization method, as discussed above). However, a discrepancy is observed for the values of $K_T^1$ between the inhibition and the binding experiments (for binding experiments, values obtained by Scatchard plots or by the optimization method are similar). Two facts may explain this difference: first, experimental conditions for binding and velocity experiments are not entirely identical (for enzymatic assay, the presence of substrates and of hydroxylamine at high concentration is required); second, we have previously observed, for some enzyme preparations, a partial desensitization toward lysine inhibition; this phenomenon appears to be more important at low protein concentration (unpublished data); this may partly explain the differences between the $K_T^1$ values, the measurement of enzyme activity being performed at much lower protein concentration than the binding experiments. (e) Moreover, the V model easily accounts for the synergistic effect displayed by lysine and leucine, the binding of both amino acids leading to the displacement toward the inactive T state of an R ⇄ T preexisting equilibrium.

Thus, all the results presented here are explained in a manner consistent with the equations of an allosteric V system.

As a matter of fact, other models such as "induced fit" model of Koshland et al. (1966) may also explain our results. However, the parallelism of Hill plots and the synergistic inhibition between lysine and leucine will require, in this model, constraining relations between the parameters not necessary in the allosteric model. Even though a definite proof of the V model hypothesis will require rapid kinetic experiments, we feel that data presented here allow us to favor it strongly at the present time.

The physiological interest of the synergistic inhibition by lysine and nonspecific amino acids may be questionable. It may be pointed out that if leucine at 0.1 mM, a concentration near the physiological one, is not an inhibitor by itself, it can enhance the lysine inhibition. In the case of aspartokinase of other species, activation or inhibition by many amino acids (usually the same as in the case of aspartokinase III: leucine, phenylalanine, valine, etc.) has been observed (Paulus and Gray, 1968; Rosner and Paulus, 1971; Dungan and Datta, 1973). The repeated occurrence of such a superimposed regulation is perhaps not without metabolic significance and may be ex-

TABLE IA: Inhibition Resulting from the Action of Two Inhibitors—Each Giving Alone 50% of Inhibition—for Different $L'$ Values.[a]

| $l$ | Inhibition for One Inhibitor (%) | Observed Inhibition (%) | $c\,(n = 2)$ | $c\,(n = 4)$ |
|---|---|---|---|---|
| 0.01 | 50 | 99 | 10 | 3.14 |
| 0.05 | 50 | 95.8 | 4.7 | 2.16 |
| 0.1 | 50 | 93 | 3.46 | 1.86 |
| 1 | 50 | 80 | 1.732 | 1.31 |
| 10 | 50 | 75.6 | 1.45 | 1.2 |

[a] Table IA gives the ratio of dissociation constants: $c = K_R/K_T$ for a dimer and a tetramer, in the case where the inhibitors alone give 50% of inhibition at saturation.

pressed as a modulation of the regulation of the main inhibitor (lysine), an example of the "metabolic interlock" as defined by Jensen (1969). From a general point of view, the physiological importance of synergistic effects in regulation is of interest; by example (see Appendix), one may obtain a quasi-complete inhibition of enzyme activity even though the binding of each inhibitor for its specific sites is not exclusive.

This may be extended to the case in which different sites exist on each protomer for the same ligand, as well as in the case of synergy between two different inhibitors, where a synergistic phenomenon may be observed which we ascribed as "autosynergy". Here again an amplification of inhibition through nonexclusive binding is possible.

Such autosynergy cannot be studied by kinetic analysis, as the binding to one site cannot be dissociated from the binding to the other one. However, it can be seen in Table I that for aspartokinase III the lysine dissociation constants toward the R and T forms, obtained by the optimization method, may express a nonexclusive binding, though a quasi-complete inhibition is observed. This may well account for an autosynergy mechanism.

The study of synergistic phenomenon is possible in any model; for example, they are very easily expressed in an allosteric model where the regulation is expressed through terms of the form: $(1 + F/K_T^F)/(1 + F/K_R^F)$ ($F$ is the ligand concentration).

The synergistic effects appear when such terms are raised to a power (cooperativity of one ligand associated with the oligomeric characteristic of most allosteric enzymes), or when they are multiplied by similar terms for other ligands (true synergy), or for the same ligand possessing more than one site per protomer (autosynergy).

In all cases the process is always the same and, as pointed out by Monod (1967 and 1968), is expressed as a magnification of chemical signals.

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## Appendix

Using eq 5 for an allosteric V system, it can be seen that a synergistic effect may lead to a quasi-total inhibition through the binding of two ligands that inhibit only partially the enzyme activity. This partial inhibition is due in this case to a nonexclusive binding to the inactive T form (expressed by the value of $c = K_R/K_T$).

Supposing that inhibitors X and Y give separately 50% of inhibition (i.e., $V_X/V_0 = V_Y/V_0 = 0.5$), the residual activity in the presence of both inhibitors is:

$$\frac{V}{V_0} = \frac{L'(0.25)}{0.25 + L'} = \frac{L'}{1 + 4L'}$$

Table IA gives the observed inhibitions as a function of $L'$ and, in each case, the different values of $c$ for a dimeric or a tetrameric protein; $c$ has been calculated from eq 1, that becomes at saturation of one inhibitor (Monod et al., 1965):

$$\frac{1 + L'}{1 + L'c^n} = \frac{V_X}{V_0} = \frac{V_Y}{V_0} = 0.5$$

($n$ being the number of protomers of the enzyme).

By example, in the case of an $L'$ value of 0.1, 93% of inhibition is obtained with a tetrameric protein for a $K_R/K_T$ value for each ligand as low as 1.86.

## References

Dungan, S. M., and Datta, P. (1973a), *J. Biol. Chem. 248,* 8534–8540.

Dungan, S. M., and Datta, P. (1973b), *J. Biol. Chem. 248,* 8541–8546.

Funkhouser, J. D., Abraham, A., Smith, V. A., and Smith, W.G. (1974), *J. Biol. Chem. 249,* 5478–5484.

Jensen, R. A. (1969), *J. Biol. Chem. 244,* 2816–2823.

Koshland, D. E., Jr., Nemethy, G., and Filmer, D. (1966), *Biochemistry 5,* 365–385.

Lafuma, C., Gros, C., and Patte, J. C. (1970), *Eur. J. Biochem. 15,* 111–115.

Monod, J., Wyman, J., and Changeux, J. P. (1965), *J. Mol. Biol. 12,* 88–118.

Monod, J. (1967 and 1968), College de France lectures.

Patte, J. C., and Cohen, G. N. (1964), *C. R. Hebd. Seances Acad. Sci. 259,* 1255–1258.

Patte, J. C., Loviny, T., and Cohen, G. N. (1965), *Biochim. Biophys. Acta 99,* 523–530.

Patte, J. C., Truffa-Bachi, P., and Cohen, G. N. (1966), *Biochim. Biophys. Acta 128,* 426–439.

Patte, J. C., Lebras, G., and Cohen, G. N. (1967), *Biochim. Biophys. Acta 136,* 245–257.

Paulus, H., and Gray, E. (1968), *J. Biol. Chem. 243,* 1349–1355.

Richaud, C., Mazat, J. P., Gros, C., and Patte, J. C. (1973), *Eur. J. Biochem. 40,* 619–629.

Richaud, C., Mazat, J. P., Felenbock, B., and Patte, J. C. (1974), *Eur. J. Biochem. 48,* 147–156.

Rosner, A., and Paulus, H. (1971), *J. Biol. Chem. 246,* 2965–2971.

Stadtman, E. R., Cohen, G. N., Lebras, G., and de Robichon Szulmajester, H. (1961), *J. Biol. Chem. 236,* 2033–2038.

Swann, W. H. (1969), *FEBS Lett. 2 Suppl.* S39–S55.

Truffa-Bachi, P., and Cohen, G. N. (1966), *Biochim. Biophys. Acta 113* 531–541.

Wampler, D. E., and Westhead, E. W. (1968), *Biochemistry 7* 1661–1671.