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pH-DEPENDENT INTERMEDIATE PLATEAUX IN THE KINETICS OF THE REACTION CATALYZED BY "BIOSYNTHETIC" L-THREONINE DEHYDRATASE OF *ESCHERICHIA COLI* K-12

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SUMMARY

1. A method for the determination of L-threonine dehydratase (L-threonine hydro-lyase (deaminating), EC 4.2.1.16) activity, based on measurements of the amount of 2-oxobutyrate formed in the threonine dehydratase reaction by means of an auxiliary lactate dehydrogenase system (EC 1.1.1.27), was described.

2. The kinetics of the reaction catalyzed by the "biosynthetic" L-threonine dehydratase in a crude extract from *Escherichia coli* K-12 cells were studied. Four inflexion points (*i.e.*, two intermediate plateaux) on the plot of initial reaction rate (v) *versus* initial substrate concentration ($[S]_0$) were distinctly revealed at pH > 9.

3. The allosteric inhibitor L-isoleucine, at certain pH values, displaced the sigmoid shape of the plot of v *versus* $[S]_0$ to higher substrate concentrations; the magnitude of the maximal reaction rate (V) did not change. At high concentrations of enzyme protein, L-isoleucine, at low concentrations, removed the intermediate plateau on the plot of v *versus* $[S]_0$ and hence "activated" the enzyme in the region of high substrate concentrations, whilst the second intermediate plateau was demonstrated in these curves as in the usual sigmoidal plots.

4. DL-Norvaline "normalized" the kinetics of the reaction catalyzed by the enzyme and at the same time decreased the value of V.

5. Treatment of the enzyme with low concentrations of $HgCl_2$ ($7.5 \cdot 10^{-5}$ M) led to the complete desensitization of the enzyme in relation to the inhibitory effect of L-isoleucine at pH 9.6 and "normalized" the effects of DL-norvaline on the reaction kinetics. The plot of v *versus* $[S]_0$ for the desensitized enzyme was characterized by the absence of the inflexion points (intermediate plateaux) which were characteristic for the native enzyme at this pH value, although the plot did not become hyperbolic. Treatment of the enzyme at pH 8.2 with lower concentrations of $HgCl_2$ ($1.0 \cdot 10^{-5}$ M) led to selective desensitization, namely, the sensitivity to the "normalizing" effect of DL-norvaline was completely lost but the sensitivity to the inhibitory effect of L-isoleucine was partially restored. It was suggested that the enzyme molecule possesses two spatially divided binding sites for these allosteric effectors.

6. The shape of the plot of *v versus* $[S]_0$ depends on the concentration of enzyme protein, namely, at low enzyme concentrations two intermediate plateaux were revealed on the plot of *v versus* $[S]_0$. At the same time, an increase in enzyme concentration led to the annihilation of the intermediate plateau demonstrated at low substrate concentrations and to the displacement of the second intermediate plateau found at high substrate concentrations towards lower substrate concentrations.

7. Low concentrations of L-isoleucine removed this second intermediate plateau only at high enzyme concentrations, and hence "activated" the enzyme at about the substrate concentrations at which the second intermediate plateau was revealed.

8. The data obtained were discussed, taking into consideration the possible effects of allosteric ligands on the dissociation of the enzyme oligomer and on the conformation states of subunits in the oligomer itself.

INTRODUCTION

L-Threonine dehydratase (L-threonine hydro-lyase (deaminating), EC 4.2.1.16) catalyzes the dehydration of L-threonine to 2-oxobutyrate and NH_3. The biosynthetic isozyme is the first enzyme of the L-isoleucine biosynthetic chain in microorganisms and plants, and is under negative feed-back control by L-isoleucine[1]. A study of this pyridoxal phosphate enzyme in *Escherichia coli* by Changeux[2-7] was one of the experimental bases for development of a general theory for the allosteric regulation of enzymatic activity[8].

However, as shown by an analysis of the literature[3,4,9], one of the fundamental characteristics of allosteric enzymes, *i.e.* the shape of the plots of initial reaction rate (v) *versus* initial substrate concentration ($[S]_0$), for the enzyme from *E. coli* was studied for an insufficiently broad range of substrate concentrations and also in a pH range (from pH 7.0 to pH 8.0) which was lower than the pH optimum (pH 9.5).

Reinvestigation of the kinetic characteristics, namely, *v versus* $[S]_0$ and *v versus* initial inhibitor concentration ($[I]_0$) in a much broader concentration range of L-threonine and L-isoleucine, respectively, and also at all pH values at which the enzymatic activity may be accurately measured, was one of the purposes of the present paper.

Kinetic studies of desensitized forms of allosteric enzymes, apart from the direct evidence of the allosteric nature of the enzymes[10], may give important information on the structure and mechanism of function of these regulatory enzymes. In this connection, and also bearing in mind the unusual kinetic characteristics of the enzyme under study, the second purpose of the present investigation was to study the kinetic behavior of this enzyme desensitized to the allosteric effectors.

At the present time it is becoming more and more evident that in the analysis of kinetic patterns of regulatory enzymes that it is necessary to take into account their capacity for reversible dissociation controlled by allosteric ligands. Systematic study of the kinetic behavior of dissociating regulatory enzyme at various concentrations of enzyme protein[11] may reveal the kinetic effects connected with the displacement of the equilibrium between the oligomeric enzyme forms under the in-

fluence of allosteric ligands. These forms differ by their degrees of aggregation, *i.e.* by molecular weights, and by their specific catalytic activities. To account for the possible contribution of dissociation effects on the unusual kinetic patterns of the enzyme under study, the effects of protein enzyme concentration on the shape of the plots of v *versus* $[I]_0$ also were investigated.

MATERIALS AND METHODS

Enzyme extract

E. coli K-12 cells were cultivated in 700 ml conical vessels on a rocking apparatus (220 oscillations/min) for 18 h at 30 °C. The volume of medium was 100 ml. The medium was inoculated with a 0.5% (v/v) 24-h culture on an agar slope. The medium contained: 3% $Na_2PO_4 \cdot 12H_2O$, 0.23% KH_2PO_4, 0.04% $MgCl_2 \cdot 6H_2O$, 0.4% $(NH_4)_2SO_4$, 2.10^{-6} M $FeSO_4 \cdot 7H_2O$ and 0.8% glucose. The medium was neutralized to pH 7.0 with a few drops of conc. H_2SO_4 and was sterilized at 1 atm for 30 min.

The cells after cultivation were collected by centrifugation at 7500 × g for 15 min at 4 °C. The cells were washed with $1 \cdot 10^{-5}$ M potassium phosphate buffer (pH 8.2). The cell paste was suspended in a 10-fold volume (per fresh wt) of 1.0 M potassium phosphate buffer (pH 8.2) which contained $1 \cdot 10^{-3}$ M reduced glutathione. The cells (40 ml of suspension) were disrupted by ultrasonic treatment (MSE-100 Desintegrator, England) for 3 min at 0–4 °C. The undamaged cells were separated by centrifugation at 14 500 × g for 15 min at 4 °C. The supernatant was used as the enzyme source.

Measurement of the enzymatic activity

Our modification of the method for the estimation of L-threonine dehydratase activity involved measuring the amount of 2-oxobutyrate formed as a result of the L-threonine dehydratase reaction by means of a lactate dehydrogenase (EC 1.1.1.27) system after the first reaction was stopped.

The reaction mixture for the L-threonine dehydratase assay contained 0.2 ml of the enzyme extract, 10 μg of pyridoxal 5'-phosphate (Fluka, Switzerland) per 1 ml, suitable amounts of L-threonine (Reanal, Hungary), and potassium–sodium–phosphate–carbonate buffer having suitable pH values; the final buffer concentration was 0.25 M; the final volume of the reaction mixture was 3.0 ml. In all experiments except those carried out to study the shape of the plot of v *versus* $[S]_0$, depending on the enzyme concentration the amount of the enzyme extract added to the reaction mixture was adjusted to equalize the enzyme activity under analogous conditions of measurement. The reaction was carried out for 5 or 10 min at 26 °C. The reaction was stopped by adding 0.075 ml of conc. H_2SO_4 followed by heating for 5 min in a boiling water bath. The precipitate was separated by centrifugation.

2-Oxobutyrate formed as a result of the reaction was measured by an enzymatic method using a preparation of muscle lactate dehydrogenase (Reanal, Hungary). For this purpose 2.5 ml of the supernatant was neutralized with 10 M KOH. 0.5 M phosphate buffer (pH 6.5) was added in order to achieve the total volume of 5 ml; the final pH value of the samples was from 6.5 to 6.7. To the neutralized samples 0.1 ml of NADH (Reanal, Hungary) solution (16.5 mg/ml) was added. From this mixture two (2.2 ml) aliquots were taken. To one of the aliquots (experimental

sample) 0.05 ml of lactate dehydrogenase suspension was added. The second sample, to which we added of 0.05 ml of buffer, was used as the control. Both samples were incubated for 15 min at room temperature. Consumption of NADH was measured by following the decrease in absorbance at 340 nm. The absorbance measurements were carried out after obtaining a constant difference between the absorbances of the control and experimental samples. A molar extinction coefficient of NADH equal to $6.22 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ was used for calculating the amount of NADH consumed (which corresponded to the amount of 2-oxobutyrate contained in the reaction mixture). All dilutions mentioned above were taken into account in the calculation of the L-threonine dehydratase specific activity. The contents of possible lactate dehydrogenase substrates in the experimental samples were considered; control experiments without L-threonine were carried out, and the absorbance values were substracted from the difference between the absorbances of the control and experimental samples mentioned above.

Special experiments at three substrate concentrations (L-threonine at $3 \cdot 10^{-3}$ M, $1 \cdot 10^{-2}$ M and $4 \cdot 10^{-2}$ M) at pH 8.2 and 9.6 and at various enzyme protein concentrations showed that the plots of *v versus* incubation time (*t*) have linear regions, the length of which depended on the substrate and enzyme concentrations. The calculation of the initial reaction rate (*v*) was carried out on the basis of the linear regions on the *v versus t* plots.

It was also observed that lactate dehydrogenase at pH 6.5 quantitatively (about 95–98%) reduced 2-oxobutyrate added to the reaction mixture (from 0.03 to 0.60 mole). The absolute error for assays of L-threonine dehydratase activity for 10 min, was $\pm$ 0.005 absorbance units (the average from 9 experiments) at pH 9.6 and at all used substrate concentrations ($2.50 \cdot 10^{-3}$ M, $1.33 \cdot 10^{-2}$ M and $6.00 \cdot 10^{-2}$ M). Thus, at high reaction rate (*i.e.* at high substrate concentrations) the relative error was 1–2%, and at the lowest reaction rate (*i.e.* at the lowest substrate concentrations) it was 8%.

Protein was determined as described by Lowry *et al.*[12]. The unit of L-threonine dehydratase activity (*i.e.* the unit of the initial reaction rate) was defined as the amount of enzyme which catalyzed the formation of 1 μmole of 2-oxobutyrate per min at 26 °C; the specific activity (*v*) was defined as the amount of the unit per mg of protein.

L-Isoleucine Koch Light (England) and DL-norvaline (U.S.S.R.) were used.

Since the enzyme preparations we used were crude extracts from *E. coli* cells, special experiments were necessary to determine the absence of "side" enzymatic reactions which may transform L-threonine (the substrate for L-threonine dehydratase) and 2-oxobutyrate (the product of L-threonine dehydratase reaction). It was observed that *E. coli* cell-free extract at pH 9.6 (0.25 M phosphate–carbonate buffer), at least during a 10-min period, did not catalyze a transformation of 2-oxobutyrate. Indeed, the incubation of sodium 2-oxobutyrate (Fluka, Switzerland) at concentrations of $1.0 \cdot 10^{-4}$ M, $3.25 \cdot 10^{-4}$ M and $7.6 \cdot 10^{-4}$ M in the buffer in the absence as well as in the presence of the cell-free extracts led in both cases to equal final concentrations of 2-oxobutyrate, as determined by the lactate dehydrogenase method described above. The possible consumption of the L-threonine added to the cell-free extracts was determined by means of a non-specific method based on the absorbance of this amino acid in the region of 225–235 nm. For this purpose, into the spectro-

photometer cuvette containing 0.25 M phosphate–carbonate buffer (pH 9.5) were added 0.2 ml of *E. coli* cell-free extract, L-threonine (at the final concentration of $1.0 \cdot 10^{-2}$ M or $5.0 \cdot 10^{-2}$ M), pyridoxal 5′-phosphate (10 μg/ml) and L-isoleucine (at the final concentration of $6 \cdot 10^{-2}$ M, *i.e.* at a concentration fully inhibiting the activity of "biosynthetic" L-threonine dehydratase). The total volume of the reaction mixture was 3.0 ml. The second spectrophotometer cuvette with the same ingredients but containing boiled extract instead of native cell-free extract was used as a control. The absorbance after 10 min of incubation at 26 °C in both cuvettes in the region of 225–235 nm were equal. Thus, in those conditions the L-threonine added to the cell-free extract was not consumed as a result of "side" enzymatic reactions for at least 10 min.

RESULTS

Dependence of reaction rate on substate concentration at different pH values

The concentration of H^+ is a potent factor which induces, apart from other effects, changes in the conformation of protein molecules. We studied therefore the effect of variation of the pH value on the shape of the plots of the initial rate (v) of the reaction catalyzed by "biosynthetic" L-threonine dehydratase against the initial substrate concentration ($[S]_0$). As shown in Fig. 1 at pH 7.1 and 8.2 the v *versus* $[S]_0$ plots had a typical sigmoidal shape. At other pH values used (*i.e.* at pH 9.1, 9.6,

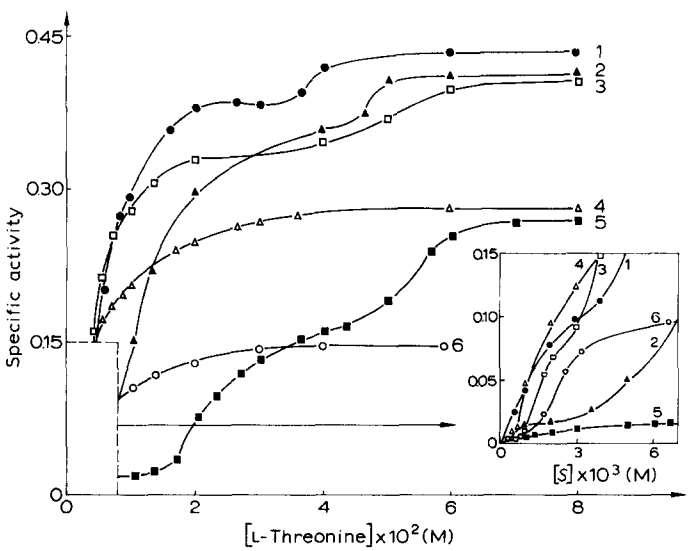

Fig. 1. Dependence of the rate of reaction catalyzed by the "biosynthetic" L-threonine dehydratase from *E. coli* K-12 on the concentration of the substrate (L-threonine) in the absence of effectors at different pH values. 1, pH 9.6; 2, pH 9.8; 3, pH 9.1; 4, pH 8.2; 5, pH 10.0; 6, pH 7.1.

9.8 and 10.0) these plots were quite different from usual sigmoidal curves; the former curves had distinct inflexion points (*i.e.* intermediate plateaux appeared on the plots of v *versus* $[S]_0$). At pH 9.6, 9.8 and 10.0, four inflexion points were distinctly revealed, and at pH 9.1 at least two inflexion points were demonstrated. (As "an inflexion

point" we considered a point on the *v versus* $[S]_0$ plot for which the second derivative of the reaction rate was equal to zero).

It was considered important to study the dependence of the reaction rate on substrate concentration at different pH values in the presence of allosteric effectors. As seen in Fig. 2, the plot of *v versus* $[S]_0$ in the absence of the effectors at pH 8.2 had a typical sigmoidal shape, *i.e.* a concave plot in Lineweaver–Burk's coordinates (Fig. 2B); values of the inconstant exponent for substrate concentration (*i.e.* inconstant Hill's coefficient of cooperativity) (n_H) were higher than unity in the broad interval of saturation of the enzyme by the substrate (Fig. 2C). The inconstant ex-

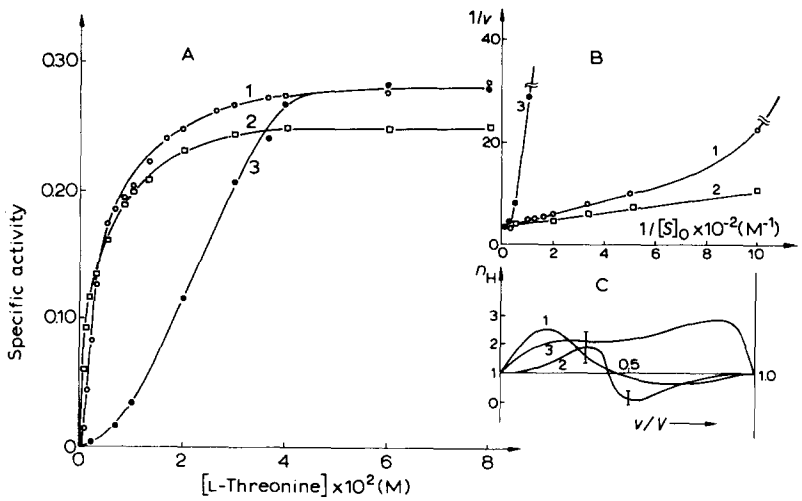

Fig. 2. Dependence of the rate of the reaction catalyzed by the "biosynthetic" L-threonine dehydratase from *E. coli* K-12 on substrate concentration at pH 8.2 in the absence and in the presence of effectors. (A) Data represented in coordinates of v (specific activity of the enzyme determined by initial reaction rate) *versus* $[S]_0$ (initial concentration of L-threonine). (B) The same in double reciprocal coordinates .(C) Dependence of inconstant exponent for substrate concentration (n_H) calculated by the "differential" Kurganov method at $x = 2$ saturation degrees of the enzyme by the substrate (*i.e.* on the ratio v/V). 1, the same in the absence of the allosteric effectors; 2, the same in the presence of DL-norvaline ($5 \cdot 10^{-3}$ M); 3, the same in the presence of L-isoleucine ($2 \cdot 10^{-4}$ M). In (C) the maximal calculation errors of the n_H values are shown by the vertical lines.

ponent for substrate concentration was calculated by the "differential" Kurganov method[13]; errors of determination of the magnitudes of n_H were calculated by the method described by Kurganov *et al.*[14]. For the calculation of the magnitude of n_H by the graphic Kurganov method the following formula was used:

$$n_H = \frac{\log \left(\frac{1}{v'} - \frac{1}{v}\right) \Big/ \left(\frac{1}{v} - \frac{1}{v''}\right)}{\log x}$$

where v, v' and v'' are reaction rates at substrate concentrations equal to $[S]_0$, $[S]_0/x$ and $x''[S]_0$, respectively, x is a constant multiplier which was higher than unity. If the empiric Hill's equation[15] is not applicable to the case, the magnitude of n_H then

depends on the substrate concentration and therefore the calculation of the value of n_H becomes a purely formal procedure. However, this inconstant exponent for substrate concentration may be used for the characterization of the shape of the plots of v *versus* $[S]_0$ as described before[16,17].

In the presence of a competitive allosteric inhibitor (L-isoleucine) (in these and the following experiments the compound used was at the final concentration of $2 \cdot 10^{-4}$ M) the sigmoidal part of the kinetic plot was markedly displaced towards higher substrate concentrations, but the maximal reaction rate (V) did not change.

DL-Norvaline (in these and the following experiments it was used at the final concentration of $5 \cdot 10^{-3}$ M) which was usually considered as an allosteric activator for "biosynthetic" L-threonine dehydratase[1], in this case decreased the magnitude of V. This amino acid activated the enzyme at low substrate concentration only, and in this way "normalized" the reaction kinetics. Indeed, the double reciprocal plot of v *versus* $[S]_0$ was a straight line (Fig. 2), and the inconstant exponent for substrate concentration $n_H \approx 1$ (within the limits of the determination errors).

At pH 9.6 the distinct plateau of v *versus* $[S]_0$ plot (Fig. 3) in absence of the effectors at substrate concentrations of $2 \cdot 10^{-2}$ to $4 \cdot 10^{-2}$ M was revealed. The occurrence of the plateau on this curve was shown by the complicated character of the plot of reaction rate *versus* substrate concentration in Lineweaver–Burk coordinates (Fig. 3B) and by the significant decrease of the inconstant exponent for substrate concentration n_H to about unity with the ratio of $v/V \approx 0.80$ (Fig. 3C), *i.e.* by kinetic indications of "negative" cooperativity[18]. A second plateau appeared in the region of substrate concentrations around $5 \cdot 10^{-3}$ M. (It was more clearly revealed at higher pH values, as shown in Fig. 1). It was associated with a decrease of $n_H < 1$

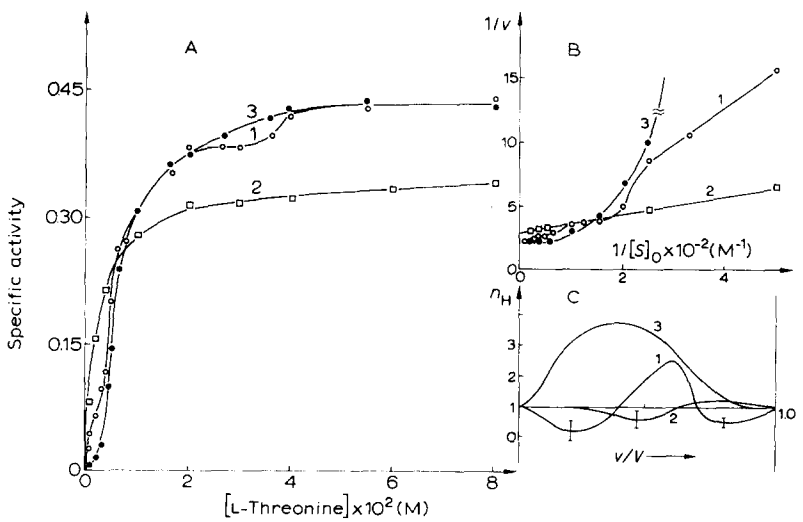

Fig. 3. Dependence of the rate of the reaction catalyzed by the "biosynthetic" L-threonine dehydratase from *E. coli* K-12 on substrate concentration at pH 9.6 in the absence and in the presence of allosteric effectors. Designations as in Fig. 2.

at saturation of the enzyme by the substrate to about 25% (the ratio of $v/V \approx 0.25$). L-Isoleucine caused less distinct effects on the reaction kinetics at this pH value than

it did in less alkaline media. This was characteristic for the "biosynthetic" L-threonine dehydratase from the bacteria under study[4]. However, the presence of L-isoleucine at low substrate concentrations led to the appearance of a sigmoidal region on the plot of v *versus* $[S]_0$ (*cf.* in Fig. 3C the magnitudes of $n_{H,max}$ for the Curves 1 and 3 in the region of the ratio of $v/V \approx 0.25$) but did not change the value of V and, quite unexpectedly, L-isoleucine removed the intermediate plateau on this plot. This character of the effect of L-isoleucine on the reaction kinetics was reflected in the concave shape of the double reciprocal plot (Fig. 3B) and in the value of $n_H > 1$ almost throughout the entire range of magnitudes for the ratio of v/V (see Fig. 3C).

At pH 9.6, as well as at pH 8.2, DL-norvaline "normalized" the reaction kinetics by more intensively decreasing the value of V at high substrate concentrations.

At pH 10.0, the plot of the reaction rate *versus* the substrate concentration (Fig. 4) in the absence of the effectors showed two distinct plateaux (*i.e.* four inflexion

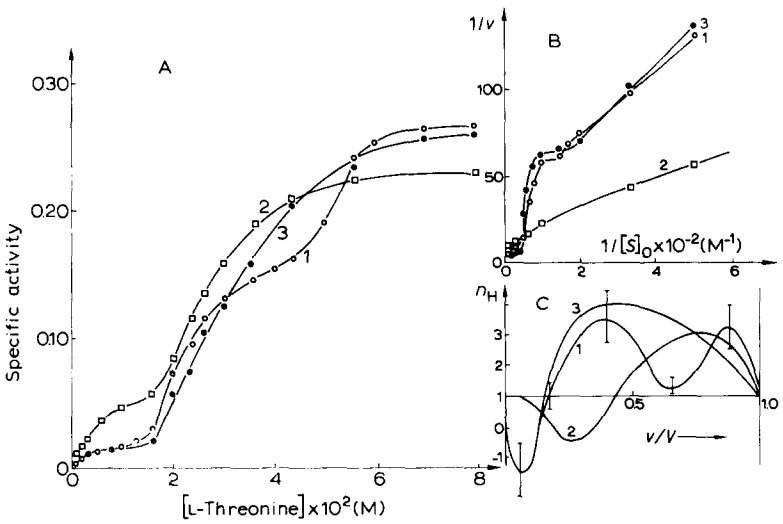

Fig. 4. Dependence of the rate of the reaction catalyzed by the "biosynthetic" L-threonine dehydratase from *E. coli* K-12 on substrate concentration at pH 10.0 in the absence and in the presence of allosteric effectors. Designations as in Figs 2 and 3.

points) which was shown by the complicated shape of this plot in Lineweaver–Burk coordinates (Fig. 4B) and in the sharp decrease of n_H for two saturation values of the enzyme by the substrate (at the ratio of $v/V \approx 0.08$ the true value of $n_H < 0$ and at the ratio of $v/V \approx 0.65$ the true value of $n_H \approx 1$). It is interesting to note that both intermediate plateaux at this pH value became more distinct and were displaced towards higher substrate concentrations as compared to the kinetic plots obtained for less alkaline medium. At pH 10.0, L-isoleucine ($2 \cdot 10^{-4}$ M) practically did not inhibit the enzyme at any substrate concentration and removed the intermediate plateau found at high substrate concentrations but did not influence the plateau observed at low substrate concentrations; this fact was reflected in the appearance of only one region of "negative" cooperativity (Fig. 4C). DL-Norvaline

($5 \cdot 10^{-3}$ M) at this pH value activated the enzyme at low substrate concentrations by decreasing, but not completely removing in this case, the first intermediary plateau (which was reflected in the appearance of "negative" cooperativity at the ratio of $v/V \approx 0.25$; Fig. 4C). This amino acid (like L-isoleucine) removed the intermediate plateau found at high degrees of saturation of the enzyme by the substrate, and inhibited the enzyme, *i.e.* decreased the value of V.

A study of the reaction kinetics at pH > 10.0 may lead to erroneous conclusions due to instability of the enzyme at very high pH values. Indeed, at pH 10.3 the enzyme was "activated" by L-isoleucine in a rather broad range of substrate concentrations (to $3 \cdot 10^{-2}$ M) which was probably caused by stabilization of the enzyme by this negative allosteric effector[1].

It is uncertain whether the unusual pattern of the kinetic plots (*i.e.* occurrence of four inflexion points on the plot of reaction rate against substrate concentration) was due to the fact that the enzyme was unpurified (crude cell-free extracts of *E. coli* were used); similar patterns were also obtained at pH 10.0 for partially purified enzyme. In this case the enzyme was purified about 10-fold by the precipitation of nucleoproteins from the cell-free extract with protamine sulfate, by subsequent fractionation of the proteins with $(NH_4)_2SO_4$ and desalting by gel filtration through Sephadex G-25.

As seen in Fig. 1, the patterns of the dependence of the reaction rate on substrate concentration were markedly changed by variation of the pH values; the shape of the plots of reaction rate *versus* pH value was, thus, different at various substrate concentrations. It seemed to be more correct to examine the dependence of the maximal reaction rate (V) on pH in order to determine the pH optimum. At saturating substrate concentrations the L-threonine dehydratase under study had a pH optimum in the region of pH 9.6 (Fig. 5) which is characteristic of "biosynthetic" L-threonine dehydratase from different organisms[1].

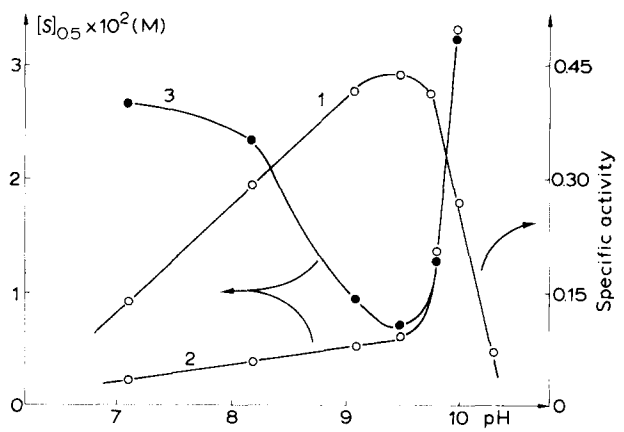

Fig. 5. Effect of pH on the activity of "biosynthetic" L-threonine dehydratase from *E. coli* K-12. 1, dependence of the specific activity of the enzyme on pH at "saturation" substrate concentration ($8 \cdot 10^{-2}$ M L-threonine); 2, dependence of the value of "semisaturation" ($[S]_{0,5}$) on pH in the absence of allosteric effectors; 3, dependence of the value of "semisaturation" ($[S]_{0,5}$)on pH in the presence of L-isoleucine ($2 \cdot 10^{-4}$ M)

For nonhyperbolic kinetic plots the reciprocal magnitude of $[S]_{0.5}$ (*i.e.* the reciprocal value of "semisaturation" of the enzyme by the substrate) is an integral characteristic of the affinity of the enzyme for the substrate. Fig. 5 shows that, in absence of the allosteric inhibitor (L-isoleucine), the integral affinity of the enzyme for the substrate was only slightly changed in the range of pH values from pH 7.0 to pH 9.6; it decreased sharply at pH values higher than the pH optimum. This phenomenon was probably due to a sharp alteration in the ionization state at pH $>$9.6 of either the functional group in the enzyme active site responsible for binding the substrate or the coenzyme (pyridoxal 5'-phosphate) or in the ionization of the functional groups in the coenzyme itself. The ionization of NH_2 and COOH groups in the substrate (L-threonine) may also influence the shape of the plot of $[S]_{0.5}$ *versus* pH.

In presence of L-isoleucine, the integral affinity of the enzyme for the substrate sharply increased with the shift of pH value from pH 7.0 to pH 9.6 (Fig. 5) and determined the decrease in the inhibitory effect of this amino acid with the increase in pH value. The sharp decrease in the integral affinity of the enzyme for the substrate in presence of L-isoleucine at pH $>$ 9.6 was, probably, determined by the same factors which were operative in absence of L-isoleucine. Consideration of the mechanism of function of the enzyme under study by means of the analysis of the dependence of integral affinity of the enzyme for the substrate (*i.e.* by the analysis of the reciprocal value of $[S]_{0.5}$) on pH value was not quite adequate. This analysis did not account for the observed patterns of the kinetic plots of v *versus* $[S]_0$ having several inflexion points. Nevertheless, it seems that the difference of the values of $[S]_{0.5}$ in the presence and in the absence of L-isoleucine at the same pH value (cf., the Curves 3 and 2 in Fig. 5) may help to evaluate the contribution of conformational transitions induced by L-isoleucine and which influence the binding of the substrate to the enzyme molecule (*i.e.*, heterotropic interaction). We supposed that the allosteric inhibitor induced the most distinct conformational changes in the enzyme molecule at pH 7.0; these conformational transitions sharply decreased in the range of pH 8 to pH 9, and the allosteric inhibitor at pH $\geqslant$ 9.6 at a given L-isoleucine concentration ($2 \cdot 10^{-4}$ M) did not induce the conformational changes which could influence the binding of the substrate with active sites on the enzyme molecules, *i.e.* it did not lead to heterotropic interactions between active and allosteric sites. The mechanism of the different effects of L-isoleucine at different pH values on the conformational transitions in the enzyme which influenced the binding of the substrate, probably consisted of the creation of obstacles to L-isoleucine binding with allosteric enzyme sites when the pH value increased, accompanied, perhaps, by changes in the ionization state of SH groups. (The role of free SH groups in the allosteric functions of L-threonine dehydratase has been reviewed[1]).

Dependence of the reaction rate on the concentration of the allosteric inhibitor

As had been shown above, L-isoleucine abolished the intermediate plateaux on the plot of initial reaction rate against initial substrate concentration, and therefore "activated" the enzyme at certain substrate concentrations (Figs 3 and 4).

At pH 9.6 low L-isoleucine concentrations ($1.0 \cdot 10^{-4}$–$1.5 \cdot 10^{-4}$ M) activated the enzyme at two substrate concentrations (3.10^{-2} M and 5.10^{-2} M) but did not activate it at an intermediate substrate concentration ($1.66.10^{-2}$ M) (Fig. 6A). On comparison of these results with the ones shown in Fig. 3 it appeared that this "activation" was

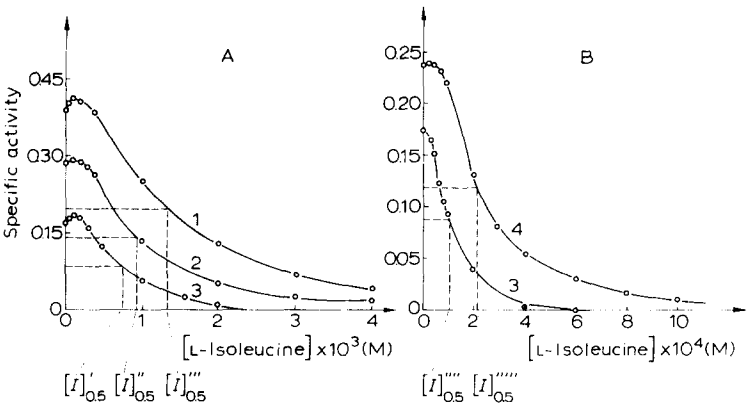

Fig. 6. Dependence of the activity of the "biosynthetic" L-threonine dehydratase from *E. coli* K-12 on the concentration of L-isoleucine at different substrate concentrations. A, pH 9.6; B, pH 8.2. The concentrations of L-threonine were: 1, $3 \cdot 10^{-2}$ M; 2, $1.66 \cdot 10^{-2}$ M; 3, $5 \cdot 10^{-3}$ M; 4, $2.66 \cdot 10^{-3}$ M; $[I]'_{0.5} = 7.0 \cdot 10^{-4}$ M; $[I]''_{0.5} = 9.5 \cdot 10^{-4}$ M; $[I]'''_{0.5} = 1.4 \cdot 10^{-3}$ M; $[I]''''_{0.5} = 9.8 \cdot 10^{-5}$ M; $[I]'''''_{0.5} = 2.2 \cdot 10^{-4}$ M.

due to the removal of the intermediate plateau on the *v versus* $[S_0]$ plot by L-isoleucine. Activation of the "biosynthetic" L-threonine dehydratase from *E. coli* by L-isoleucine at low substrate concentrations was also observed by Sanchez and Changeux[19].

At pH 8.2 when the intermediate plateaux on the plot of v against $[S]_0$ was absent, the enzyme was not activated by L-isoleucine (Fig. 6B).

When L-isoleucine did not activate the enzyme, the plots of the dependence of reaction rate (v) on the concentration of allosteric inhibitor ($[I]_0$) were sigmoidal (Fig. 6). This suggested the occurrence of several allosteric sites on the enzyme and homotropic cooperative interactions between them. The inhibition had a competitive character in respect to the substrate; an integral affinity of the enzyme for the inhibitor (*i.e.* the reciprocal value of $[I]_{0.5}$ which was equal to the inhibitor concentration inhibited the enzyme up to 50% at a given substrate concentration) was decreased by an increase in the substrate concentration. In full conformity with the data presented in Figs. 2 and 3, the integral affinity of the enzyme for L-isoleucine at pH 8.2 was higher than at pH 9.6 (*cf.* the values of $[I]_{0.5}$ in Figs 6A and 6B).

Kinetic study of the desensitized enzyme

Unusual patterns of the plot of *v versus* $[S]_0$, *i.e.* the appearance of several inflexion points on this curve, were clearly seen only at pH > 9. Reaction kinetics for a desensitized form of this enzyme from *E. coli* were, however, studied[5] only at pH 8.1. It was found that the plot of *v versus* $[S]_0$ obtained by heat desensitization or by desensitization by means of treatment with $HgCl_2$ was "normalized", *i.e.* it was transformed from a sigmoidal shape to a hyperbolic one. These data also allowed the enzyme to be considered according to the allosteric model of Monod *et al.*[8]. In this connection we considered it important to study the kinetics of the reaction catalyzed by the desensitized enzyme at pH > 9.

We used treatment with low concentrations of $HgCl_2$ as a desensitizing pro-

cedure. Cell-free extract from *E. coli* K-12 was adjusted to equivalent catalytic activity (as compared with above mentioned experiments) and was incubated at pH 8.2 for 30 min at 0–4 °C in the presence of $HgCl_2$ (final concentration was $1 \cdot 10^{-5}$ M or $7.5 \cdot 10^{-5}$ M). The enzymatic activity was then determined in the absence as well as in the presence of L-isoleucine ($2 \cdot 10^{-4}$ M) or DL-norvaline ($5 \cdot 10^{-3}$ M) at various L-threonine concentrations.

After this treatment the enzyme was completely desensitized at pH 9.6: the plots of v *versus* $[S]_0$ in the absence and in the presence of both allosteric effectors overlapped (Fig. 7). Kinetic plots for the desensitized enzyme lost the inflexion

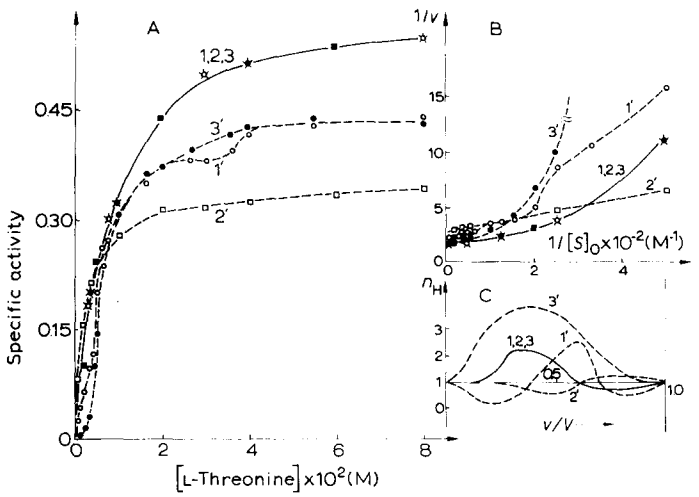

Fig. 7. Dependence of the rate of the reaction catalyzed by "biosynthetic" L-threonine dehydratase from *E. coli* K-12 in the native and $HgCl_2$-desensitized ($7.5 \cdot 10^{-5}$ M $HgCl_2$) forms on substrate concentration at pH 9.6. (A) Data presented in v *versus* $[S]_0$ coordinates. (B) The same data in double reciprocal coordinates. (C) Dependence of the inconstant exponent for substrate concentration n_H on the degree of saturation of the enzyme by the substrate (*i.e.* on the ratio of v/V). 1 and 1′, desensitized and native enzymes, respectively; the activities were measured in the absence of the effectors; 2 and 2′, desensitized and native enzymes, respectively; the activities were measured in the presence of DL-norvaline ($5 \cdot 10^{-3}$ M); 3 and 3′, desensitized and native enzymes, respectivily; the activities were measured in the presence of L-isoleucine ($2 \cdot 10^{-4}$ M).

points on the curve of v *versus* $[S]_0$ plots which were characteristic for the native enzyme. At the same time, this plot did not acquire a hyperbolic shape (Figs 7B and 7C). Indeed, the double reciprocal plot (see Fig. 7B) was concave with respect to the abscissa, which indicated its deviation from the hyperbolic shape. In the range of substrate saturation from 0.25 to 0.50 (Fig. 7B), for this plot the magnitude of inconstant exponent for substrate concentration n_H is significantly higher than unity ($n_{H,max} \approx 2$), which was also characteristic for nonhyperbolic plots.

During the desensitization process under these conditions, the enzyme was activated, *i.e.* the maximal reaction rate (V) increased in comparison with the native enzyme. Similar results were obtained for "biosynthetic" L-threonine dehydratase from strains *E. coli* K-12 and IHM 544-*Su*-1 by Freundlich and Umbarger[20] and for

a similar enzyme from subcellular structures of pea seedlings by Tomova *et al.*[21]. The mechanism of enzyme activation during its desensitization by low concentrations of $HgCl_2$ probably consisted of changes in the conformation of enzyme molecule during the binding of SH groups, thus leading to an increase in the dissociation rate of an enzyme-substrate complex, *i.e.* to an increase in the kinetic constant k_2.

Since at pH 9.6 the enzyme under study was much less sensitive to the inhibitory effect of L-isoleucine than at lower pH values, we decided to obtain a completely desensitized modification of the enzyme under conditions of high sensitivity to L-isoleucine, for example, at pH 8.2. Under these conditions Hg^{2+} ($7.5 \cdot 10^{-5}$ M) also completely desensitized the enzyme to both allosteric effectors (Fig. 8). In this

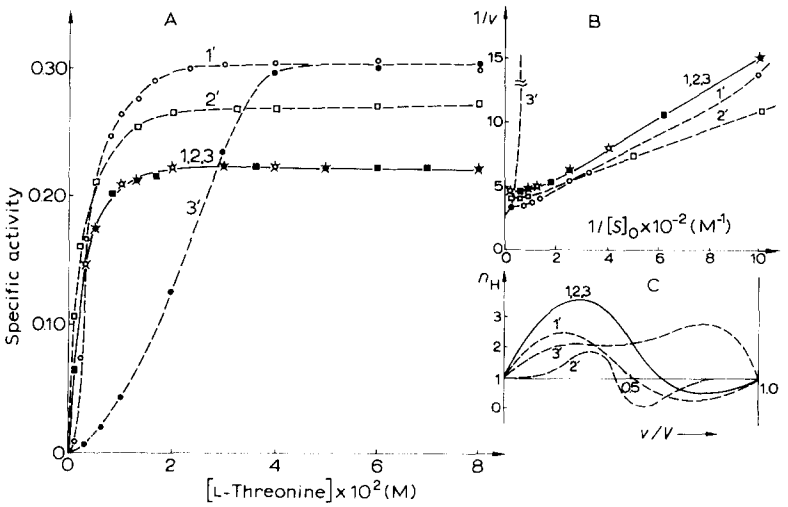

Fig. 8. Dependence of the rate of the reaction catalyzed by the "biosynthetic" L-threonine dehydratase from *E. coli* K-12 in the native and $HgCl_2$-desensitized ($7.5 \cdot 10^{-5}$ M $HgCl_2$) forms on substrate concentration at pH 8.2. Designations as in Fig. 7.

case the desensitization was not accompanied by "normalization" of the kinetics of the reaction catalyzed by the enzyme. This was indicated by the experimental data plotted in double reciprocal coordinates where the curve of v *versus* $[S]_0$ was neither sigmoidal nor hyperbolic (Fig. 8B). In agreement with these data the inconstant exponent for substrate concentration n_H was higher than unity in the range of 0.1 to 0.6 complete saturation of the enzyme by the substrate. But in this case the enzymatic activity decreased, *i.e.* the value of V decreased, probably due to partial inactivation of the enzyme by Hg^{2+}.

In relation to the above mentioned partial enzyme inactivation, we tried to desensitize the enzyme by means of lower concentrations of Hg^{2+}. At $1 \cdot 10^{-5}$ M $HgCl_2$ the enzyme was activated, *i.e.* the value of V for the treated enzyme was higher than for the native enzyme (Fig. 9). Under these conditions the enzyme completely lost its sensitivity to DL-norvaline (*cf.* plots 2 and 2' in Fig. 9A) but was only partially desensitized to the inhibitory effect of L-isoleucine (*cf.* Plots 3 and 3' in the same Fig. 9A). In presence of L-isoleucine the plot of v *versus* $[S]_0$ for partially desensitized

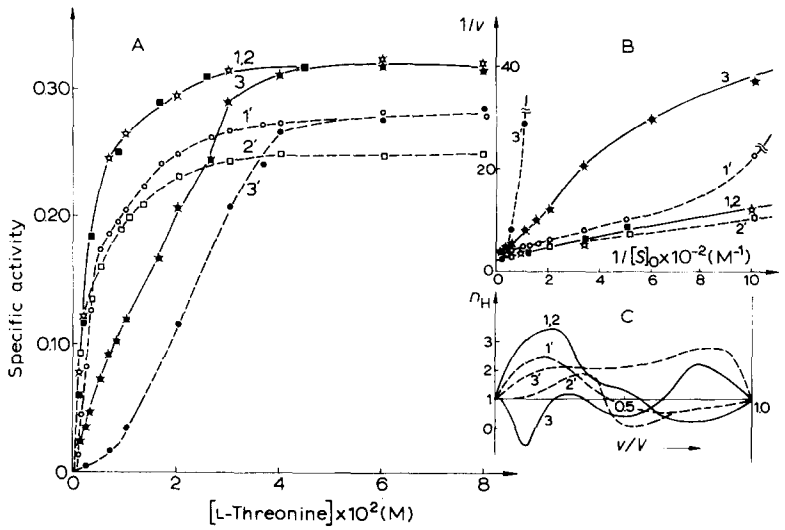

Fig. 9. Dependence of the rate of the reaction catalyzed by the "biosynthetic" L-threonine dehydratase from *E. coli* K-12 in the native and $HgCl_2$-desensitized ($1 \cdot 10^{-5}$ M $HgCl_2$) forms on substrate concentration at pH 8.2. Designations as in Fig. 7.

enzyme was transformed from a sigmoidal curve to a more complicated shape; this was reflected in the appearence of "negative" cooperativity. Indeed, the magnitude of the inconstant exponent for substrate concentration n_H was significantly lower than unity in the 0.2 region of complete saturation of the enzyme by the substrate (Fig. 9C); the double reciprocal plot had a concave shape (Fig. 9B).

This selective desensitization with respect to two allosteric effectors indicated that the binding sites for the allosteric inhibitor (L-isoleucine) and the binding sites for the second allosteric ligand (DL-norvaline, possessing an ability to "normalize" the reaction kinetics) were spatially divided in the molecule of *E. coli* K-12 "biosynthetic" L-threonine dehydratase. This conclusion is in agreement with that reached by Changeux[22], but in contradiction with the works of Freudenlich and Umbarger[20] and of Hardings *et al.*[23] who assumed that both effectors have a single allosteric site in the enzyme.

Effect of protein concentration on the allosteric properties of the enzyme

The unusual kinetic patterns of "biosynthetic" L-threonine dehydratase from *E. coli* K-12 (*i.e.* the appearance of two intermediate plateaux on the *v versus* $[S]_0$ plot) observed by us may be consistent with the "indirect" cooperativity (sequential) model of Koshland[24]. They may be explained[25] on the basis of the assumption that, in the oligomeric enzyme, in the course of the sequential saturation of the active sites by substrate the allosteric interactions between the active sites (*i.e.* the magnitudes of microscopic binding constants and (or) microscopic catalytic constants) initially decreased, followed by a subsequent increase. However, the tetrameric model of Koshland[25] does not explain the occurrence of the second plateau on the plot of *v versus* $[S]_0$. Indeed, it should be supposed in this case that the enzyme under study

consisted of more than four subunits, in agreement with the unmodified model of Koshland.

At present there is no indication of the subunit structure of "biosynthetic" L-threonine dehydratase from *E. coli*. However, it had been shown that the analogous enzyme from the closely related bacterium *Salmonella typhimurium*[26,27] consists of four subunits and contains only two active sites (*i.e.* two pyridoxal 5′-phosphate binding sites). On the other hand, the similar enzyme from the bacterium *Rhodospirillum rubrum*[28] has a tetrameric structure containing four moles of pyridoxal 5′-phosphate per mole of protein (mol. wt 180 000) and is able to associate to form catalytically active octamer.

An alternative explaination of the appearance of the second intermediate plateau on the *v versus* $[S]_0$ plot for the enzyme under study is an assumption that the enzyme belongs to the class of dissociating regulatory enzymes. The shape of the plots of *v versus* $[S]_0$ and *v versus* initial concentration of allosteric effectors for these enzymes depends on the enzyme concentration itself[11] as well as on allosteric sensitivity[11]. To examine this assumption, the effects of enzyme protein concentration on the shape of the plots of *v versus* $[S]_0$ and *v versus* $[I]_0$ were investigated.

The recurrent fact that at pH 9.6 the shape of the plot of *v versus* $[S]_0$ is mainly dependent on the enzyme protein concentration is shown in Fig. 10. At the given protein concentration the kinetic curve (Plot 3) had two characteristic intermediate plateaux. At the increased enzyme concentration the plateau which appeared at low substrate concentrations was lost. The second plateau (appearing at high substrate concentrations) in this case was displaced towards lower L-threonine concentrations (see Plots 1 and 2). On the other hand, at the decreased enzyme concentration (Plot 4)

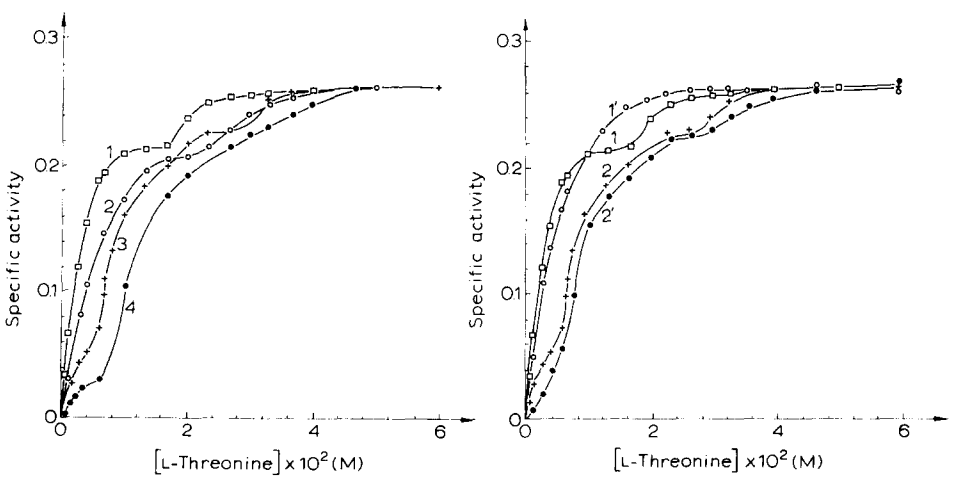

Fig. 10. Dependence of specific activity (v) of "biosynthetic" L-threonine dehydratase from *E. coli* K-12 on substrate concentration at pH 9.5 at different enzyme protein concentrations: 1, 0.500 mg/ml; 2, 0.090 mg/ml; 3, 0.028 mg/ml; 4, 0.010 mg/ml.

Fig. 11. Dependence of specific activity (v) of "biosynthetic" L-threonine dehydratase from *E. coli* K-12 on substrate concentration at pH 9.5 at two enzyme protein concentrations (Plots 1 and 1′, 0.500 mg/ml and Plots 2 and 2′, 0.028 mg/ml) in the presence (Plots 1′ and 2′) and in the absence (Plots 1 and 2) of L-isoleucine ($2 \cdot 10^{-4}$ M).

the first intermediate plateau was more clearly revealed, and the second intermediate plateau appeared over a wider range of substrate concentrations and, hence, became less distinct. It should be noted that the maximal specific enzymatic activity (V), *i.e.* the enzyme specific activity at "saturation" substrate concentration, did not depend on the enzyme protein concentration. From this fact we may derive a very significant methodological conclusion, nemaly, that a change of the specific activity of an enzyme in relation to its concentration may be demonstrated only by using substrate concentrations lower than the "saturation" level.

An analysis of the experimental data shown in the Fig. 10 suggested that the complex shape of the kinetic plots observed had no connection with the possible occurrence of some isozymes of "biosynthetic" L-threonine dehydratase in crude *E. coli* cell-free extracts, each characterized by different affinity to the L-threonine (*i.e.* different magnitudes of K_m and of catalytic constant k_2). Indeed, if the extracts contained a mixture of these isozymes, then the kinetic plots must have had the same shape at different enzyme concentrations. Thus, a more probable assumption explaining the data obtained would be an alteration in the oligomeric state of the enzyme molecule, *i.e.* the oligomer dissociates to catalytically less active subforms on dilution of the enzyme protein.

In connection with the change of the kinetic curves in relation to enzyme concentration it was interesting to investigate the sensitivity of the enzyme to the retroinhibition effects of L-isoleucine at different enzyme concentrations. At pH 9.5 (Fig. 11) and at high enzyme concentration L-isoleucine ($2 \cdot 10^{-4}$ M) removed the intermediate plateaux on the plot of v *versus* $[S]_0$ and, hence, activated the enzyme in the region of high substrate concentrations at which the second intermediate plateau is revealed; in this case the magnitude of V did not change (*cf.* Plots 1 and 1'). On the contrary, at lower enzyme concentration (*cf.* Plots 2 and 2') L-isoleucine removed the first intermediate plateau which appeared at low substrate concentration but either did not influence the second intermediate plateau in the region of high L-threonine concentrations or slightly increased its length.

A plot of relative specific enzymatic activity (v_i/v, where v_i is specific enzymatic activity in presence of L-isoleucine) against concentration of allosteric inhibitor at

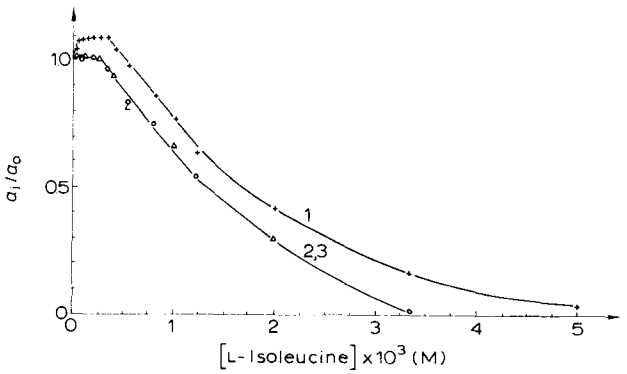

Fig. 12. Dependence of relative specific activity (v_i/v) of "biosynthetic" L-threonine dehydratase from *E. coli* K-12 on the concentration of L-isoleucine at pH 9.5 at different enzyme protein concentrations (1, 0.360 mg/ml; 2, 0.090 mg/ml; 3, 0.028 mg/ml). L-Threonine was used in concentration $1.66 \cdot 10^{-2}$ M.

three enzyme concentrations is represented in Fig. 12. It should be noted that in these experiments we used the substrate concentration at which the second intermediate plateau on the *v versus* $[S]_0$ plot appeared at two enzyme concentrations (see Fig. 11). Fig. 12 shows that at high enzyme concentration the low concentrations of L-isoleucine activated the enzyme but the plot of the ratio v_i/v *versus* L-isoleucine concentration at low enzyme protein concentrations had the typical sigmoidal shape The data obtained are in full agreement with those of the Fig. 11. It should be noted that there probably exists a threshold enzyme concentration above or below which the plot of *v versus* $[S]_0$ has a different shape.

DISCUSSION

Analysis of all the experimental data obtained led us to the following conclusions. In alkaline medium "biosynthetic" L-threonine dehydratase from *E. coli* K-12 revealed unusual kinetic patterns which were characterized by the appearance of four inflexion points (two intermediate plateaux) on the plot of initial reaction rate *versus* initial substrate concentration.

It should be noted that the *v versus* $[S]_0$ plots having similar inflexion points are known for several other enzymes: phosphoenolpyruvate carboxylase (CO_2 fixation rate *versus* acetyl-CoA concentration[29]), ADP:glucose pyrophosphorylase (*v versus* ATP concentration[30]), L-glutamate dehydrogenase coupled with NAD (dependence of the rate of oxidative deamination of L-glutamate on NAD concentration[31]), glyceraldehyde-3-phosphate dehydrogenase (*i.e.* for the plot of *v versus* NAD concentration[32]), and CTP synthetase (plot of *v versus* glutamine concentration[33]).

Similar curves with several inflexion points (*i.e.* with intermediate plateaux on the *v versus* $[S]_0$ plot) are predicted by only one modern model for allosteric enzymes, namely, by Koshland's "direct" cooperativity (sequential) model[24,25]. The phenomenon may be explained[25] on the basis of an assumption that in an oligomeric protein, in course of sequential saturation of active sites by the substrate, the allosteric interactions between the active sites initially decrease and this is followed by a subsequent increase.

The extremely complex shape of the *v versus* $[S]_0$ plots, the change of the shape of these plots depending on the enzyme protein concentration, and the different sensitivities of the enzyme to the inhibitory effects of L-isoleucine at various enzyme concentrations show that, when analyzing the obtained data it is necessary to take into account the contribution of effects connected with the influence of allosteric effectors on the reversible dissociation of the enzyme. The complex shape of the kinetic plots suggests, in our opinion, the existence of several intermediate forms of the enzyme during dissociation. The substrate displaces the equilibrium between these oligomeric forms in consistence with the model of dissociating regulatory enzymes[11]. The displacement of the equilibrium under the influence of the substrate in this case leads to the formation of the more catalytically active (and, perhaps, higher molecular weight) form. This conclusion is derived from the existence of the unchanging magnitude of V at various enzyme protein concentrations.

It should be emphasized that the complex character of the *v versus* $[S]_0$ plots (*i.e.* the occurrence of two intermediate plateaux) is impossible to explain by the

model of dissociating regulatory enzymes only, if it is assumed that the probable structure of the enzyme is tetrameric, even taking into consideration the fact that there are several intermediate catalytically active enzyme subforms. As has been noted, the subunit structure of "biosynthetic" L-threonine dehydratase from *E. coli* is unknown at present. However, the similar enzyme from *S. typhimurium*[26,27] consists of four subunits but bears only two active sites, and the enzyme from *R. rubrum*[28] is a tetramer capable of associating to form catalytically active octamers.

It may be assumed that any model capable of explaining all the obtained experimental data, among them the appearance of the second intermediate plateau on the *v versus* $[S]_0$ plots, the "normalizing" effect of DL-norvaline on the reaction kinetics, and the effect of L-isoleucine in removing the intermediary plateau on the *v versus* $[S]_0$ plots even for tetrameric enzyme, is a "hybrid" model. This model must contain elements of Koshland's model[24] and of the dissociating regulatory enzyme model[11], *i.e.* it must be a model which takes into consideration conformational transitions of two types: in the oligomer itself and reversible dissociation of the oligomer.

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