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Wheat-Germ Aspartate Transcarbamoylase

KINETIC BEHAVIOUR SUGGESTING AN ALLOSTERIC MECHANISM OF REGULATION

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1. Some kinetic properties of aspartate transcarbamoylase (EC 2.1.3.2), that had been purified approx. 20-fold from wheat germ, were studied. 2. A plot of enzyme activity against pH showed a low maximum at pH 8.4 and a second, higher, maximum at pH 10.5. A plot of percentage inhibition by 0.2mM-UMP against pH was approximately parallel to the plot of activity against pH, except that between pH 6.5 and 7.5 the enzyme was insensitive to 0.2mM-UMP. 3. Kinetics were studied in detail at pH 10.0 and 25°C. In the absence of UMP, initial-rate plots were hyperbolic when the concentration of either substrate was varied. UMP decreased both $V_{max.}$ and K_m in plots of initial rate against L-aspartate concentration, but the plots remained hyperbolic. However, UMP converted plots of initial rate against carbamoyl phosphate concentration into a sigmoidal shape, without significantly affecting $V_{max.}$. Plots of initial rate against UMP concentration were also sigmoidal. 4. The theoretical model proposed by Monod *et al.* (1965) gave a partial explanation of these results. When quasi-equilibrium conditions were assumed analysis in terms of this model suggested a trimeric enzyme binding the allosteric ligands, carbamoyl phosphate and UMP, nearly exclusively to the R and T conformational states respectively, and existing predominantly in the R state when ligands were absent. However, the values of the Hill coefficients for the co-operativity of each allosteric ligand were somewhat less than those predicted by the theory. 5. Some of the implications of these results are discussed, and the enzyme is contrasted with the well-known aspartate transcarbamoylase of *Escherichia coli*.

Aspartate transcarbamoylase (EC 2.1.3.2) catalyses an early step in the pathway of biosynthesis *de novo* of pyrimidine nucleotides, namely: L-aspartate + carbamoyl phosphate → *N*-carbamoylaspartate + P_i. In *Escherichia coli* the enzyme is inhibited by a number of end-products of the pathway, notably CTP. This inhibition is known to play an important role in the metabolic regulation of the pathway (Yates & Pardee, 1956; Gerhart & Pardee, 1964). Because the purified enzyme is available in gram amounts, it has been a favourite regulatory enzyme for experimental studies, and an extensive literature on this enzyme now exists (see Gerhart, 1970, for a recent review). However, the regulatory properties of the *E. coli* enzyme are not shared by the aspartate transcarbamoylases of most other organisms. Bethell & Jones (1969) have summarized studies on the enzyme from representative species of some of the major groups of living organisms. These and subsequent studies have shown that there is considerable phylogenetic variation in the properties of the enzyme. In a majority of the species that have been studied, the enzyme does not appear to be directly implicated in feedback control of pyrimidine nucleotide biosynthesis.

Among higher plants, aspartate transcarbamoylase is known to be inhibited *in vitro* by UMP, in extracts of lettuce seedlings (Neumann & Jones, 1962, 1964), mung-bean seedlings (Ong & Jackson, 1970), and wheat germ (Yon, 1970). These findings suggested that in higher plants the enzyme has a regulatory function. Preliminary studies on the wheat-germ enzyme revealed a number of important differences between this enzyme and other regulatory aspartate transcarbamoylases that have been described in some detail, such as those of *E. coli* (Gerhart, 1970), other bacteria (Bethell & Jones, 1969) and yeast (Lue & Kaplan, 1969). The wheat-germ enzyme is noteworthy since it combines regulatory behaviour with the comparatively small mol.wt. of approx. 100000 (Yon, 1970). No other regulatory aspartate transcarbamoylase with a mol.wt. of less than 300000 has been reported.

The experiments described in the present paper were undertaken to find out whether kinetic evidence for an allosteric mechanism of inhibition could be

obtained. Allosteric interactions (Monod *et al.*, 1965) are a property of many regulatory enzymes, including the aspartate transcarbamoylase of *E. coli* (Gerhart, 1970). If such interactions could be demonstrated for the wheat-germ enzyme, the mechanism would have to involve a smaller protein than the *E. coli* enzyme.

A preliminary report of this work has been published (Yon, 1971).

Experimental

Chemicals

L-Aspartic acid (chromatographically homogeneous) was obtained from British Drug Houses Ltd., Poole, Dorset, U.K. Carbamoyl phosphate (dilithium salt), carbamoylaspartic acid and UMP (disodium salt) were products of Sigma Chemical Co., St. Louis, Mo., U.S.A. All other chemicals used as reagents or buffers were of analytical-reagent grade.

A sample of the carbamoyl phosphate was shown to be 92% pure and to contain less than 5% of P_i, by determining the phosphate content before and after complete acid hydrolysis. It was used without further purification. During routine use, solutions of carbamoyl phosphate were made up to 15mM in water and stored immediately at −15°C in 5ml portions. These were thawed not more than 5min before use and were diluted with water as required.

Enzyme preparation

Aspartate transcarbamoylase was extracted from commercial wheat germ (Bemax; Vitamins Ltd., Brentford, Middx., U.K.). This was defatted by extraction with three changes of diethyl ether at room temperature, each being just sufficient to cover the settled wheat germ. The defatted dried wheat germ was mechanically ground to a powder. The powder (400g) was suspended in 2 litres of water and stirred for 2h at room temperature. Coarse material was removed by centrifugation. All centrifugations were carried out in the 6×250ml rotor of the MSE High-Speed 18 centrifuge for 20min at 10000*g*. The supernatant was heated rapidly to 60°C in a large Pyrex flask, which was kept at this temperature for 5min before being rapidly cooled by swirling the flask in ice-cold water. All subsequent operations, including centrifugation, were performed at 5°C or below. Denatured protein was removed by centrifugation. Acetone, previously cooled to −15°C, was added over a 5min period with stirring; 380ml of acetone were added for every 1000ml of heat-treated supernatant. The mixture was kept at −8°C in an ice–salt bath for 2h. The precipitate was then removed by centrifugation and discarded. The acetone treatment was repeated on the supernatant, a further 210ml of cold acetone being used to every 1000ml of the previous heat-treated supernatant. After 2h at −8°C the precipitate was separated by centrifugation, and then was resuspended in ice-cold 10mM-imidazole buffer, previously adjusted to pH7.5 with 2M-HCl. The final volume was one-tenth of that of the heat-treated supernatant. All lumps were broken up by using a hand-operated tissue homogenizer. Any material remaining undissolved after standing overnight at 4°C was removed by centrifugation and discarded. The clear brown supernatant was stored at 4°C and was used in the experiments described below. It was diluted with water just before use.

Enzyme assay

The following assay method was used during the partial purification of the enzyme. The reaction mixture contained 2.4ml of buffered aspartate (1.0mM-L-aspartate in 0.2M-glycine, adjusted to pH10.0 with 10M-NaOH), 0.2ml of water, 0.2ml of enzyme extract (diluted with water, if necessary) and 0.2ml of 15mM-carbamoyl phosphate in water. The reaction was started by the addition of the carbamoyl phosphate, and was stopped after 10min at 25°C by adding 0.5ml of 4M-$HClO_4$. After removal of precipitated protein by centrifugation, carbamoylaspartate in the clear supernatant was determined by the colorimetric method of Prescott & Jones (1969). Control experiments to compensate for endogenous or non-enzymically formed ureido compounds were performed as a routine. As the original aqueous extract contained a heat-stable inhibitor of aspartate transcarbamoylase, portions of the original and heat-treated supernatants were passed through small columns of Sephadex G-25 before assay.

Protein was determined colorimetrically (Lowry *et al.*, 1951) with bovine serum albumin (fraction V; Sigma Chemical Co.) dried to constant weight at 60°C as standard.

Kinetic experiments

All kinetic experiments were performed at 25°C. Details of concentrations of substrates and inhibitors are given with the results of each experiment. For experiments in which the pH was varied, a mixed buffer system was used consisting of 0.1M each of imidazole, tris and glycine. L-Aspartate was dissolved in this mixture, and the whole was adjusted with small volumes of 10M-HCl, 10M-NaOH or water to give buffered aspartate solutions at a series of pH values, with L-aspartate at a final concentration of 12.5mM and each buffer component at a concentration of 0.090–0.095M. The reaction mixture contained 2.4ml of buffered aspartate, 0.2ml of 15mM-carbamoyl phosphate in water, 0.2ml of water or 3.0mM-UMP in water, and 0.2ml of diluted enzyme preparation. The final pH was determined with a glass electrode and pH-meter while the reaction was in progress.

All other kinetic experiments were performed at pH10.0. Glycine and L-aspartate were dissolved in water and adjusted with 10M-NaOH to pH10.0 and final concentrations of 30mM-L-aspartate and 0.1M-glycine. Various concentrations of aspartate were obtained by diluting this solution with 0.1M-glycine, pH10.0. Reaction mixtures contained 2.4ml of buffered aspartate to which were added 0.2ml each of carbamoyl phosphate, UMP or water, and diluted enzyme, to the desired final concentrations. In some of the experiments at low concentrations of carbamoyl phosphate, these volumes were all increased 2.5-fold, to give sufficient liquid to fill a 4cm-light-path cuvette (see below).

A reaction time of 5min was used in all the kinetic experiments, to limit the extent of the base-catalysed decomposition of carbamoyl phosphate at pH values above 9 (Allen & Jones, 1964). The enzyme preparation was diluted 5-fold in water before use, to ensure that the carbamoylaspartate produced in this time was 10% or less of the theoretical yield (the reaction is essentially irreversible). Trial experiments showed that the production of carbamoylaspartate was linear with time up to 15% of the theoretical yield.

Most reactions were carried out in a total volume of 3.0ml, and were stopped by adding an equal volume of the colorimetric reagent mixture (Prescott & Jones, 1969). Carbamoylaspartate was determined by the shorter of the two methods described by these authors. Because of the low K_m for carbamoyl phosphate, however, a more sensitive method was needed at low concentrations of this substrate. In these reactions the total reaction volume was increased to 7.5ml, and the reaction was stopped by adding 7.5ml of the colorimetric reagent mixture. The yellow colour was developed by the longer and more sensitive of the two methods described by Prescott & Jones (1969) and the extinction measured in 4-cm light-path cuvettes. By this method it was possible to determine 1μM-carbamoylaspartate in the reaction mixture with sufficient accuracy.

In all experiments, appropriate controls were performed to compensate for a very slight turbidity that occurred after addition of the colorimetric reagent, and for carbamoylaspartate produced non-enzymically. Experiments were performed in duplicate.

Analysis of sigmoid kinetic data

The kinetic data of Fig. 3 were fitted to the Hill equation by the iterative method described by Wieker *et al.* (1970). The computer program for this purpose was kindly supplied by Dr. H.-J. Wieker, and was adapted for the University of London CDC 6600 computer by Mr. J. R. Dunlop.

Results

Stability and purity of the enzyme preparation

Table 1 summarizes a typical partial purification of aspartate transcarbamoylase. Enzyme activity and susceptibility to inhibition by UMP were both very stable on storage at 4°C; in one case over 70% of the initial activity was present after 12 weeks of storage, and the residual activity was still strongly inhibited by UMP. The preparation darkened in colour within the first few days, and was periodically recentrifuged to remove protein impurities that gradually precipitated. On the basis of the distribution of protein and activity in gel-filtration experiments (Yon, 1970), the enzyme comprised less than 5% of the protein in the fresh preparation.

Incubation of the preparation with 0.5mM-L-glutamine, 50mM-$NaHCO_3$, 30mM-$MgSO_4$, 7.5mM-ATP and 10mM-L-aspartate, in 0.2M-tris–HCl buffer, pH7.5, for 1h led to no detectable production of carbamoylaspartate when the enzyme was used at concentrations similar to those in kinetic experiments. The preparation was therefore free of carbamoyl phosphate synthetase activity at pH7.5. Incubation of the preparation with 1.0mM-carbamoylaspartate for 1h at pH7.5 led to no detectable decrease in carbamoylaspartate. The preparation was therefore also free of dihydro-orotase activity at pH7.5. Incubation of the preparation with carbamoyl phosphate alone led to a small increase in free phosphate, which could not be accounted for solely as a

Table 1. *Partial purification of aspartate transcarbamoylase from commercial wheat germ*

One unit of enzyme activity is defined as the amount that will catalyse the formation of 1μmol of carbamoylaspartate/min under the standard enzyme assay conditions (see the Experimental section).

Stage	Protein (g)	Total activity (units)	Specific activity (units/g)	Yield (%)	Purification
Water extract	57.6	223	3.9	100	1
Supernatant after heat treatment at 60°C	33.5	199	5.9	89	1.51
Redissolved acetone precipitate	2.3	175	76	78	19.6

result of the non-enzymic decomposition of carbamoyl phosphate. The preparation therefore probably contained a small amount of phosphatase activity. The effect of this activity was negligible at the enzyme concentrations and incubation times used in the kinetic experiments reported below.

Dependence of activity and inhibition on pH

Changes in pH were found to affect both catalytic activity and susceptibility to inhibition by UMP. The pH–activity relationship is shown in Fig. 1. In the absence of UMP, the substrate concentrations used (10mM-aspartate and 1.0mM-carbamoyl phosphate) were probably sufficiently high to saturate the active site at all pH values tested, thus avoiding effects on the K_m values. However, the pH profile shown is probably only an approximation of the ideal curve of $V_{max.}$ against pH, owing to substrate inhibition by 10mM-aspartate, at least near pH10. The occurrence of two maxima, one at pH8.4 and a higher one at pH10.5, is unusual. Similar pH profiles have been reported for the aspartate transcarbamoylases of *E. coli* (Weitzman & Wilson, 1966) and mouse spleen (Hoogenraad *et al.*, 1971). Portions of wheat-germ enzyme that had been preincubated for 10min at either pH6.5 or pH10.5 yielded pH–activity profiles identical with those shown in Fig. 1, suggesting that the changes between these pH limits were fully reversible. However, enzyme assayed at pH10.0 before and after incubation for 30min at pH11.5 suffered a permanent decrease in activity, suggesting that the fall in the pH profile above pH10.5 was due to irreversible inactivation of the enzyme.

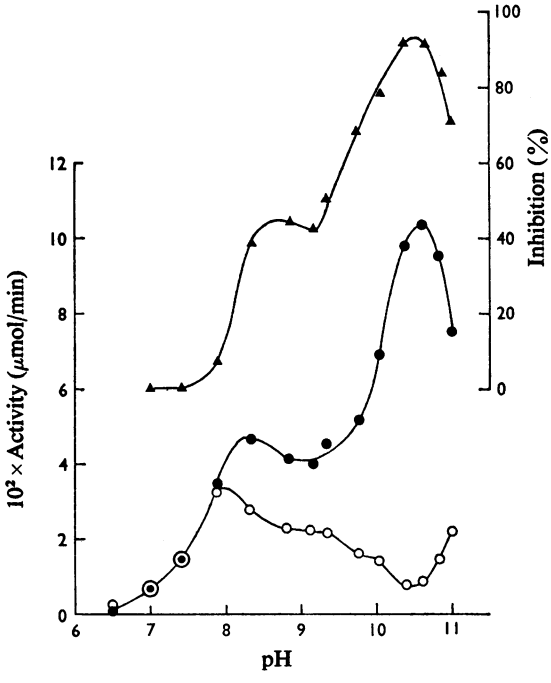

Fig. 1. *Effect of pH on enzyme activity in the absence and presence of UMP*

At each pH the reaction mixture contained 10mM-L-aspartate and 1.0mM-carbamoyl phosphate. UMP, when used, was at 0.2mM. The figure shows enzyme activity in the absence of UMP (●) and in the presence of 0.2mM-UMP (○). Also shown is the percentage inhibition by 0.2mM-UMP (▲).

Fig. 1 also shows the changes in the pH–activity relationship that are caused by including 0.2mM-UMP in the reaction mixture. Separate plots are given for residual activity and for percentage inhibition. Between pH6.5 and pH7.5 the enzyme was insensitive to 0.2mM-UMP under the stated conditions of substrate concentration. Above pH7.5 the percentage inhibition rose sharply and above pH8.0 the percentage inhibition and the catalytic activity changed in an approximately parallel manner. At pH10.5 the enzyme was at once most active and most sensitive to UMP. A single functional group may be required in the deprotonated form for maximum activity as well as maximum sensitivity to UMP, because the shapes of the activity and percentage inhibition curves both suggest the titration of functional groups with pK_a values near 10.

Effects of UMP on substrate-saturation curves at pH10.0

A detailed kinetic study was made at pH10.0 and 25°C. The variation of initial rate as a function of aspartate concentration is shown in Fig. 2. In the absence and in the presence of UMP the initial rate was a hyperbolic function of aspartate concentration (Fig. 2*a*). In the absence of UMP, the K_m for aspartate was 0.6mM. Double-reciprocal plots of the same data (Fig. 2*b*) showed that the effect of UMP was to decrease both $V_{max.}$ and the apparent K_m for aspartate. At concentrations above 1.0mM, substrate inhibition by aspartate became significant.

The corresponding kinetics with respect to the second substrate, carbamoyl phosphate, are shown in Fig. 3. In the absence of UMP the rate curve was hyperbolic; from these results the K_m for carbamoyl phosphate was 10μM. The addition of UMP converted the rate curve into a sigmoidal shape. The co-operativity of carbamoyl phosphate, as measured by the Hill coefficient (see the Experimental section) was determined at various UMP concentrations. The results are given in Table 2. The Hill coefficient (n_H) increased rapidly from 0.96 in the absence of UMP to 2.51 in the presence of 10μM-UMP. At higher concentrations of UMP, up to 1000μM, values of n_H

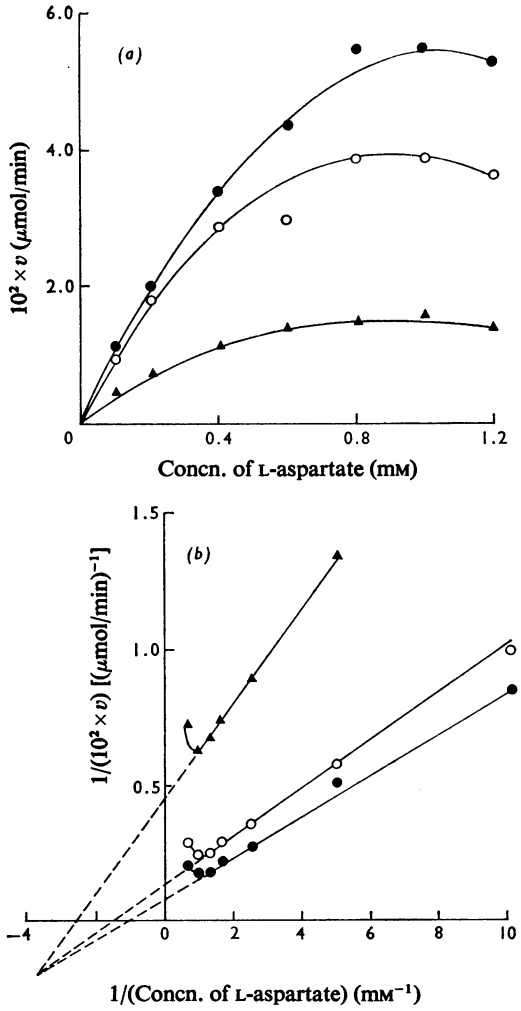

Fig. 2. *Effect of the concentration of* L-*aspartate on the initial reaction rate at pH* 10.0 *in the absence and presence of UMP*

The carbamoyl phosphate concentration was kept constant at 1.0 mM. Experiments were performed in the absence of UMP (●), and in the presence of 0.1 mM-UMP (○) and 0.2 mM-UMP (▲). The same results are shown as hyperbolic curves (*a*) and double-reciprocal plots (*b*).

were all in the range 2.6–2.8. Table 2 also shows the corresponding values of $V_{max.}$ and $[S]_{0.5}$ (the concentration of carbamoyl phosphate at which $v = 0.5\,V_{max.}$). Changes in the concentration of UMP did not significantly alter $V_{max.}$; there were only random variations in $V_{max.}$, in the range 80–120 nmol/min, although the concentration of UMP was changed by a maximum factor of 1000. However, changes in the concentration of UMP produced corresponding changes in $[S]_{0.5}$.

Plots of initial rate as a function of UMP concentration, in the presence of constant substrate concentrations, were also sigmoidal (Fig. 4). These results suggested that UMP was bound co-operatively to the enzyme in the presence of substrates. Hill coefficients for the three curves in Fig. 4 were 2.40, 2.48 and 2.55.

Interpretation of kinetic data in terms of the Monod–Wyman–Changeux model

Co-operative kinetics may, in certain cases, be satisfactorily explained by the allosteric model proposed by Monod *et al.* (1965). On the assumption that wheat-germ asparate transcarbamoylase is a symmetrical oligomer in equilibrium between two conformation states, R and T, which have preferential affinities for carbamoyl phosphate and UMP respectively, it is possible to give a satisfactory explanation of the results of Figs. 3 and 4 and Table 2. In the context of this model, carbamoyl phosphate and UMP show the typical heterotropic interaction to be expected from antagonistic allosteric ligands. The requirement that each ligand should show homotropic co-operativity in the presence of a constant concentration of the other is fully met by Figs. 3 and 4. The conformation state that binds carbamoyl phosphate (R) must also be the state of the enzyme in the absence of allosteric ligands, since there is no co-operativity in the carbamoyl phosphate rate curve in the absence of UMP. A consequence of this conclusion is that the binding of UMP must necessarily show homotropic co-operativity in the absence of carbamoyl phosphate; however, it is not possible to test this by kinetic methods. The observation that $V_{max.}$ is unaffected, within the limits of error, by changes in the concentration of UMP (Table 2) suggests that the effect of UMP is on the binding of carbamoyl phosphate only, not on its catalytic function as the carbamoyl donor, i.e. the enzyme is a 'K' system in the terminology of Monod *et al.* (1965).

For a more quantitative application of the model proposed by Monod *et al.* (1965) to the present study, estimations were made of the values of the constants in the equation of Monod *et al.* (1965) (see below), which would enable the equation to accomodate the data of Fig. 3 and Table 2. For this purpose, the quasi-equilibrium assumption (Wong & Endrenyi, 1971) was made, under which the fractional saturation (Y) may be equated with $v/V_{max.}$. The dissociation constant, K_s, between carbamoyl phosphate and the R conformation was taken to be the same as the K_m of carbamoyl phosphate. α is the 'normalized' substrate concentration, i.e. (concn. of carbamoyl phosphate)/K_s.

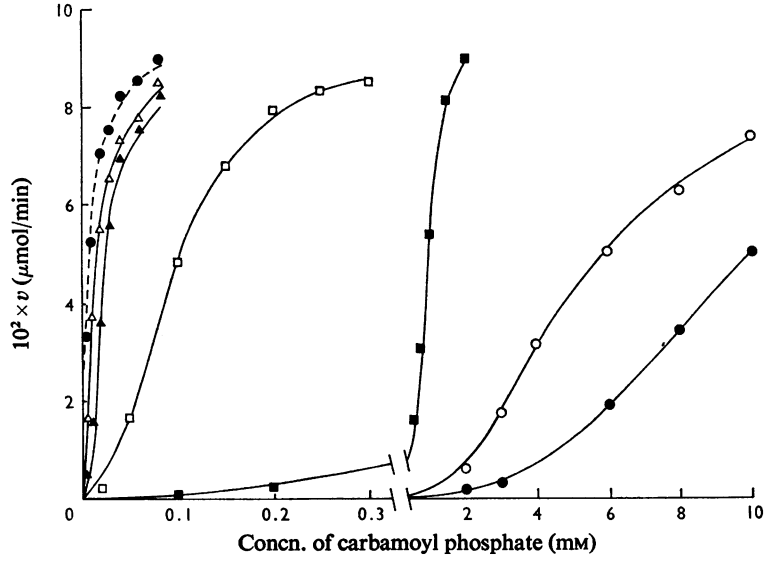

Fig. 3. *Effect of the concentration of carbamoyl phosphate on initial reaction rate at pH* 10.0 *in the absence and presence of UMP*

The concentration of L-aspartate was kept constant at 1.0mM. Experiments were performed in the absence of UMP (●----●) and in the presence of the following concentrations of UMP: 1μM (△); 2μM (▲); 10μM (□); 100μM (■); 500μM (○); 1000μM (●——●).

Table 2. *Analysis of the co-operativity of carbamoyl phosphate in terms of the Hill equation* $v = \frac{V_{max.}}{1+([S]_{0.5}/[S])^{n_H}}$

The values of $V_{max.}$, n_H and $[S]_{0.5}$ were generated from the data of Fig. 3 by using the iterative computer program of Wieker *et al.* (1970).

Concn. of UMP (μM)	$10^2 \times V_{max.}$ (μmol/min)	n_H	$[S]_{0.5}$ (μM)	$\log \alpha_{0.5}$*
0	9.52	0.96	10.5 (= K_m)	0
1.0	9.54	1.27	15.9	0.179
2.0	9.25	1.87	24.2	0.318
10.0	8.98	2.51	92	0.942
100	10.2	2.73	942	1.953
500	8.32	2.79	4850	2.667
1000	12.1	2.67	11380	3.033

* $\alpha_{0.5} = [S]_{0.5}/K_m$, where $[S]_{0.5}$ is the concn. of carbamoyl phosphate at which $v = 0.5\,V_{max.}$.

The equation of Monod *et al.* (1965) expresses $\bar{Y}$ as a function of α and of the constants n, L and c:

$$\bar{Y} = \frac{Lc\alpha(1+c\alpha)^{n-1} + \alpha(1+\alpha)^{n-1}}{L(1+c\alpha)^n + (1+\alpha)^n}$$

The constant c (the ratio of the affinity of carbamoyl phosphate for the T state to its affinity for the R state) was determined first, because its value determined the form of the subsequent analysis. Frieden (1967) showed that when plots are made of $\bar{Y}/\alpha$ against $\bar{Y}$, in the presence of high concentrations of the antagonistic ligand (in this case UMP), these plots tend to a limiting intercept on the $\bar{Y}/\alpha$ axis. The length of this intercept is c. Fig. 5(*a*) shows replots of the kinetic data in the presence of 500μM-UMP and 1000μM-UMP. They show that the value of c is approx. 5×10^{-5}. Thus for all practical purposes the binding

of carbamoyl phosphate was exclusive to the R state. The remainder of the analysis was made on the assumption that $c = 0$.

The constant L is defined as the equilibrium constant [T]/[R] in the absence of carbamoyl phosphate. In the general case, L is a function of the concentration of UMP. In the special case where UMP is also absent the constant is denoted by L_0 and is called the intrinsic allosteric constant. The values of L, and the number of monomers, n, were determined as described by Horn & Börnig (1969). These authors derived a linear transformation of the equation of Monod *et al.* (1965), applicable only to the exclusive-binding case, in which $\log L$ (or $\log L_0$) is given by the vertical intercept of the corresponding linear plot, and $-(n-1)$ by the slope. These plots are shown in Fig. 5(*b*). By trial, the best integral slope was found to be -2, suggesting a trimeric structure for the enzyme. With a slope of -2, lines were adjusted by eye as shown in Fig. 5(*b*) and the intercepts read off. The corresponding values of L are given in Table 3. Confidence in the values found for n and L was increased when it was found that an alternative method of estimation, the curve-fitting method of Frieden (1967), gave substantially the same results.

At low values of α in the vicinity of the K_m for carbamoyl phosphate, the Horn & Börnig (1969) plots were too inaccurate to be useful. Accordingly, the allosteric constant L_0 and the dissociation constant K_t between UMP and the T state were estimated by the following alternative approach. First it was assumed that UMP bound exclusively to enzyme in the T state. This was suggested by the observation that at sufficiently high concentrations of UMP, the reaction rate could always be decreased, apparently to zero (given the detection limit set by the assay method). Under the alternative assumption, that UMP had appreciable affinity for enzyme in the R state, there would always be some residual activity, however high the UMP concentration was. Secondly, it was assumed that no activators of the enzyme were present (i.e. allosteric ligands other than carbamoyl phosphate with affinity mainly for enzyme in the R state). Under these conditions, the value of the UMP-dependent constant, L, for a trimeric enzyme would be given by the following equation (also after Monod *et al.*, 1965):

$$L = L_0(1 + [\mathrm{UMP}]/K_t)^3$$

By a process of trial and error, it should therefore be possible to find values for the constants L_0 and K_t which, when substituted in this equation, give a series of values of L that correspond as closely as possible to the values found experimentally (i.e. by the method of Horn & Börnig, 1969). This has been done in Table 3, from which it is estimated that L_0 has the value 0.1 and K_t the value 0.5μM. The agreement between calculated and experimental values of L in Table 3 is only approximate, with individual differences as large as 28%. Nevertheless the overall consistency between the two sets of values, spanning several orders of magnitude, is encouraging. The relatively small value of L_0 confirms that, in the absence of allosteric ligands, the conformational equilibrium is displaced very much in favour of the R state.

One final result bears on the behaviour of the Hill coefficient that is predicted for various theoretical models. In general, this coefficient is non-integral and is frequently used to estimate a minimum number of co-operative binding sites. Only in exceptional cases do so-called 'Hill kinetics' apply, when the coefficient has an integral value equal to the number of binding sites for a particular ligand. The dependence of the Hill coefficient on the concentration of an antagonistic ligand has been described by Rubin & Changeux (1966) for the general non-exclusive model proposed by Monod *et al.* (1965). In this case the Hill co-efficient passes through a maximum which is less than n, the number of sites (and of monomers). However, in the case of the exclusively-binding model, 'Hill kinetics' become possible in the presence of a sufficiently high concentration of the antagonistic ligand (Rubin & Changeux, 1966;

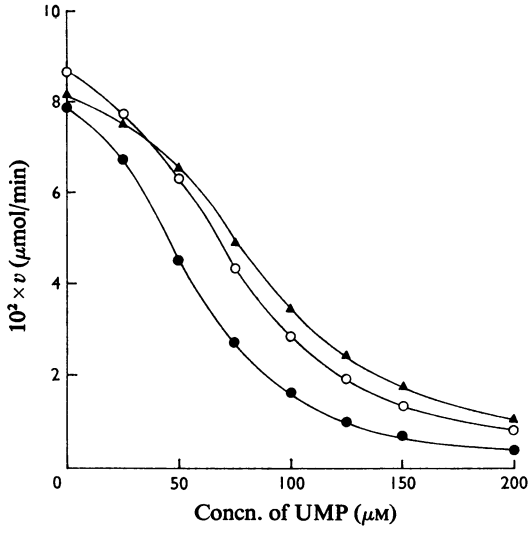

Fig. 4. *Effect of UMP on the initial reaction rate at pH* 10.0 *in the presence of various concentrations of carbamoyl phosphate*

The concentration of L-aspartate was kept constant at 1.0mM. Experiments were performed in the presence of the following constant concentrations of carbamoyl phosphate: 0.6mM (●); 0.8mM (○); 1.0mM (▲).

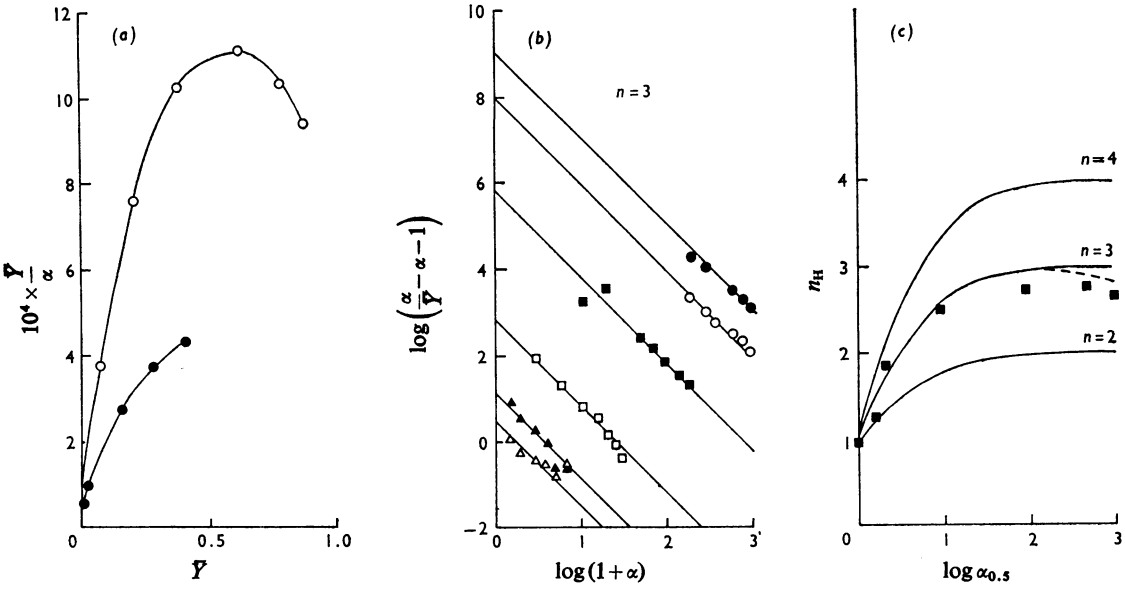

Fig. 5. *Interpretation of the kinetic interaction between carbamoyl phosphate and UMP in terms of the allosteric model of Monod et al.* (1965)

Replots of the data of Fig. 3 and Table 2 are shown. α = (concn. of carbamoyl phosphate)/K_s, where K_s is the dissociation constant of the complex between the R conformation and carbamoyl phosphate (see the text). $\bar{Y}$ is the fractional saturation of carbamoyl phosphate-binding sites. Quasi-equilibrium conditions are assumed (Wong & Endrenyi, 1971) hence $\bar{Y} = v/V_{max.}$ and $K_s = K_m$ (carbamoyl phosphate). (*a*). Modified Scatchard plots at the two highest concentrations of UMP, showing near-exclusive binding of carbamoyl phosphate to the R state (after Frieden, 1967). The value of *c*, from the vertical intercept, is 5×10^{-5}. (*b*). Estimation of the number of protomers (*n*) and UMP-dependent allosteric constants (*L*) by the method of Horn & Börnig (1969). Exclusive binding ($c = 0$) is assumed. The best integral slope was −2, corresponding to $n = 3$. The values of *L*, given by the vertical intercepts, are shown in Table 3. (*c*). Plot of Hill coefficient (n_H) against $\log \alpha_{0.5}$ (after Rubin & Changeux, 1966). The points are experimental results from Table 2. The continuous lines are theoretical, for a dimer, trimer or tetramer that binds substrate exclusively to the R conformation. The discontinuous line shows the effect of taking $c = 5 \times 10^{-5}$ instead of $c = 0$ in the case of the theoretical trimer. Theoretical values were calculated from an equation given by Buc & Buc (1968). In (*a*) and (*b*) the concentrations of UMP are as follows: 1 μM (△); 2 μM (▲); 10 μM (□); 100 μM (■); 500 μM (○); 1000 μM (●).

Whitehead, 1970). To be consistent with the previous interpretation, therefore, Hill coefficients for the co-operativity of carbamoyl phosphate should approach the value of 3 at high concentrations of UMP. Table 2 and Fig. 5(*c*) show that this is only approximately true; the highest value of n_H was 2.79, although a value of 3 would be expected over most of the range of concentrations of UMP that was tested. A similar result was obtained when it was attempted to induce 'Hill kinetics' for the co-operativity of UMP (Fig. 4), in the presence of high constant concentrations of carbamoyl phosphate. Only Hill coefficients in the range 2.4–2.6 could be obtained, although the concentration of carbamoyl phosphate was increased to 5 mM.

Discussion

pH-dependence of catalytic and regulatory functions

The physiological significance of the pH-dependence shown in Fig. 1 is not understood. Maximum catalytic activity and maximum sensitivity to end-product inhibition by UMP were both expressed *in vitro* under conditions of high alkalinity (pH 10.5). However, at near-neutral pH values only low activity and no response to UMP (at 0.2 mM) was observed. It seems unlikely that highly alkaline conditions occur to any large extent in either the developing or the dormant wheat embryo, or in the germinating seedling, and it is therefore difficult to rationalize the pH-dependence *in vitro* with the most efficient catalytic

Table 3. *Demonstration of approximate agreement between experimental and calculated values of L when the values* $L_0 = 0.10$ *and* $K_t = 0.5\,\mu$M *are used*

Experimental values were obtained from the Horn–Börnig (1969) plots (Fig. 5*b*). Calculated values were obtained by substituting the concentrations of UMP in the equation $L = L_0(1+[\text{UMP}]/K_t)^3$.

Concn. of UMP (μM)	Values of L		Experimental / Calculated
	Experimental	Calculated	
1	3.2	2.7	1.18
2	16	12.5	1.28
10	800	926	0.87
100	8×10^5	8.1×10^5	0.98
500	1.0×10^8	1.0×10^8	1.00
1000	1.0×10^9	0.8×10^9	1.25

and regulatory use of the enzyme *in vivo*. It is possible that activating conditions, at present unknown, exist in the cell, which are able to reverse the effects of neutral pH observed *in vitro*. Alternatively the enzyme may be spatially confined to an alkaline micro-environment. Further experiments are needed to evaluate these possibilities.

Allosteric interactions

Although it is theoretically possible for a single-site enzyme to show apparent homotropic co-operativity, no examples of such behaviour are known (Koshland, 1970). Further, these mechanisms have not yet been shown to explain heterotropic interactions such as those described in the present paper. For these reasons such mechanisms are considered unlikely to account for the present results. The possibility that co-operativity may arise from reversible subunit aggregation (Nichol *et al.*, 1967; Frieden, 1967) appeared unlikely after it was shown that at pH 10 and 22°C there was little difference in the elution profiles of the enzyme from Sephadex G-200 in the presence and in the absence of UMP (R. J. Yon, unpublished work). It appeared most likely, therefore, that the kinetic effects reported here were produced by a multi-site enzyme mechanism involving interactions between sites. These interactions have been most successfully explained in models assuming conformational changes.

Several conformational models have been described in recent years, and the symmetrical model of Monod *et al.* (1965) is the simplest to interpret experimentally on account of its severe restrictions on allowed conformations and on the behaviour of ligand-binding sites. As shown above, the model is capable of a partial explanation of the behaviour of wheat-germ aspartate transcarbamoylase. It suggests a trimeric enzyme having two conformational states that bind preferentially the ligands UMP and carbamoyl phosphate respectively, the latter state being also the normal conformation of the enzyme in the absence of ligands. The observed co-operativity of either ligand in the presence of the other argues strongly for a conformation mechanism, such as the model proposed by Monod *et al.* (1965). However, this co-operativity, by falling short of the theoretical value of the Hill coefficient, fails to meet rigorously the consequences of the assumption of exclusive, or near-exclusive ligand-binding (Fig. 5*c*) even though, as shown previously, there are good grounds for making this assumption.

Because L-aspartate shows no co-operativity in the presence and in the absence of UMP, it is clear that, in the terms of the model proposed by Monod *et al.* (1965), this ligand does not have the large difference in affinities for the two conformational states which the other ligands show. It is possible, however, that a small difference exists. If aspartate binds better, but not exclusively, to enzyme in the T state, this would explain both the inhibition by high concentrations of this substrate and the effect of UMP in decreasing the apparent K_m of aspartate.

It must be emphasized that none of the results described above rules out the possibility of an alternative conformational mechanism, such as the sequential mechanism of Koshland *et al.* (1966), for example. On the basis of kinetic results alone it is impossible to distinguish unambiguously between alternative conformational mechanisms, since the interpretation must rest on unproven assumptions about the applicability of kinetic measurements to models describing ligand-binding (e.g. in the present case, the quasi-equilibrium assumption). Thus the present suggestion that this enzyme is an example of the mechanism proposed by Monod *et al.* (1965) must remain tentative until its predictions on subunit and binding-site stoicheiometry, the concerted conformational transitions, and the opposing effects of UMP and carbamoyl phosphate have been independently tested.

Comparison with the aspartate transcarbamoylase of E. coli

Aspartate transcarbamoylase from *E. coli* has been studied intensively, and is also thought to be an example of the model proposed by Monod *et al.* (1965) (Gerhart, 1970). There are a number of notable contrasts between the *E. coli* and wheat-germ enzymes. In *E. coli* aspartate transcarbamoylase, the main antagonistic pair of allosteric ligands are asparate and the end-product inhibitor CTP. Equilibrium-dialysis studies have shown that succinate (an aspartate analogue) binds co-operatively to the enzyme–carbamoyl phosphate complex in the absence of CTP, whereas CTP does not bind co-operatively in the absence of other ligands. This indicates that the enzyme is predominantly in the T state (with low substrate affinity) in the absence of ligands. Thus in these two enzymes, not only is the allosteric interaction directed at different substrates, but the initial equilibrium distribution of the enzyme between the catalytically active (R) and inactive (T) states is different. Further, *E. coli* aspartate transcarbamoylase is activated by ATP (Gerhart & Pardee, 1962); so far no activators of the wheat-germ enzyme have been found, although several purine and pyrimidine nucleotides and related compounds have been tried. Finally, the inhibiting pyrimidine nucleotide is different. These differences undoubtedly reflect differences in the detailed metabolic roles of the enzyme in the two organisms.

Potentially the most interesting comparison between the two enzymes concerns the relation between structure and regulatory function. Gel filtration of wheat-germ enzyme (Yon, 1970) at pH 7.5 indicated a mol.wt. of approx. 100000; further experiments have confirmed that under conditions similar to those of the kinetic experiments described above (pH 10.0, 22°C) there is no substantial change in the gel-filtration behaviour (R. J. Yon, unpublished work). If, in addition, the enzyme is indeed a trimer as suggested by the kinetic results, then it is remarkably similar in gross structural features to the 'catalytic subunit' of the *E. coli* enzyme. This subunit also has a mol.wt. of 100000 (Gerhart & Schachman, 1965) and contains three polypeptide chains (Meighen *et al.*, 1970; Rosenbusch & Weber, 1971). It retains full catalytic activity, but entirely lacks regulatory properties (Gerhart & Schachman, 1965). If this similarity extends to details of primary structure it would have interesting evolutionary implications. However, answers to these questions on the structure of wheat-germ aspartate transcarbamoylase must await the preparation of pure enzyme.

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