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# Purification and characterization of *N*-acetylglutamate 5-phosphotransferase from pea (*Pisum sativum*) cotyledons

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*N*-Acetylglutamate 5-phosphotransferase (acetylglutamate kinase, EC 2.7.2.8) has been isolated from pea (*Pisum sativum*) cotyledons and purified 312-fold by using heat treatment, $(NH_4)_2SO_4$ fractionation, affinity chromatography on ATP–Sepharose and ion-exchange chromatography on DEAE-cellulose. This preparation was shown on polyacrylamide-gel electrophoresis to yield one band staining with Coomassie Blue. The enzyme was shown by a variety of techniques to be composed of two different kinds of subunits, of mol.wts. 43000 and 53000 respectively. These subunits are arranged to give either a dimeric or tetrameric enzyme composed of equal numbers of each type of subunit. The dimeric and tetrameric enzyme forms are thought to be interconvertible, the equilibrium between these forms being influenced by the type of ligand bound to the subunits. Kinetic studies performed on the purified enzyme, indicated a random Bi Bi type of mechanism. The enzyme displayed apparent negative co-operativity with respect to one of its substrates, *N*-acetylglutamate; as a result, two $K_m$ values were found for this substrate, one at $1.9 \times 10^{-3}$ M and the other at $6.2 \times 10^{-3}$ M. A single $K_m$ value for ATP was found to be $1.7 \times 10^{-3}$ M. Allosteric regulation by arginine was also shown. A model, based on the Koshland, Némethy & Filmer [(1966) *Biochemistry* **5**, 365–385] Sequential model, which adequately describes the kinetic and structural properties of *N*-acetylglutamate 5-phosphotransferase, is presented.

In micro-organisms, ornithine synthesis from glutamate occurs via a series of *N*-acetyl intermediates (Vogel, 1953; Cybis & Davis, 1974; Faragó & Dénes, 1967; Haas & Leisinger, 1975). More recent work has shown that certain key enzymes involved in this pathway are also found in tissues of a number of higher-plant species (McKay & Shargool, 1977; Morris & Thompson, 1977). These species have been shown to utilize a pathway in which the primary route for the synthesis of *N*-acetylglutamate involves the transfer of an acetyl group from acetylornithine to glutamate (McKay & Shargool, 1977; Morris & Thompson, 1977). In the single-celled green alga *Chlamydomonas reinhardti*, where a similar pathway occurs (Faragó & Dénes, 1967), the enzyme *N*-acetylglutamate 5-phosphotransferase (acetylglutamate kinase, EC 2.7.2.8) has been shown to be sensitive to feed-back inhibition by arginine, and presumably plays an important role in the regulation of arginine biosynthesis in this organism *in vivo*. We therefore decided to find a method of purifying this enzyme from higher-plant tissues, enabling further more complex studies to be performed.

Abbreviations used: SDS, sodium dodecyl sulphate; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

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## Materials and methods

### Chemicals

Adenosine 5′-triphosphate, adenosine-5′-diphosphate, γ-methoxyglutamate, *N*-acetylglutamate and dimethyl suberimidate were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Adenine derivatives of Sepharose 4B were obtained from P-L Biochemicals Inc., Milwaukee, WI, U.S.A. Polyvinylpyrrolidone was obtained from Chemical Developments of Canada Ltd., Montreal, Canada, and purified as described by Loomis & Battaile (1966). Adenosine 5′-[γ-thio]triphosphate and adenosine 5′-[α,β-methylene]triphosphate were obtained from Boehringer Mannheim Canada Ltd., Ville St. Laurent, Quebec, Canada. DEAE-cellulose



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(DE 52) was purchased from Mandel Scientific Co., Montreal, P.Q., Canada. All other chemicals used were of the highest commercial grade available.

*Assays*

The routine assay was based on that described by Dénes (1970), and utilized enzyme dissolved in 0.01 M-potassium phosphate buffer, pH 7.5, containing 10 mM-EDTA, 10 mM-2-mercaptoethanol and 15% (v/v) glycerol (Buffer A). Substrate concentrations were 0.1 M for *N*-acetylglutamate and 0.01 M for ATP. Incubation was at 30°C for 2h; the total reaction volume was 0.5 ml, utilizing up to 0.1 ml of enzyme (corresponding to amounts of protein of 30–230 μg). Reactions were terminated by the addition of 1.0 ml of $FeCl_3$ reagent, and the $A_{540}$ of the supernatants measured after centrifugation at 12000 ***g*** for 10 min. In these reactions 0.0143 $A \equiv 1$ μmol of *N*-acetyl-γ-glutamyl hydroxamate ($\varepsilon_{540} = 21.45$ litre·mol$^{-1}$·cm$^{-1}$). A standard curve was constructed by using *N*-acetyl-γ-glutamyl hydroxamate prepared by acetylation of γ-methoxy-L-glutamate (Miyai *et al.*, 1962) followed by treatment with hydroxylamine hydrochloride (Khedouri & Meister, 1965). The unit of enzyme activity used in the present paper is equivalent to 1 μmol of hydroxamate produced per 60 min.

*Homogenization and preliminary enzyme purification*

Seeds of pea (*Pisum sativum* L., var. Homesteader) were soaked in deionized water for 24 h, then 100 g of cotyledons were homogenized in 500 ml of 0.1 M-potassium phosphate buffer, pH 7.5, containing 1.0 mM-2-mercaptoethanol, 0.1 mM-EDTA and 15% (v/v) glycerol (Buffer B). Polyvinylpyrrolidone (100 g) was also added to this mixture. Homogenization was performed in a Waring blender at top speed for 90 s. The homogenate was squeezed through four layers of muslin and the filtrate centrifuged at 16300 ***g*** for 20 min at 4°C. The starchy layer on top of the supernatant was removed and the supernatant adjusted to 1 mM-*N*-acetylglutamate by the addition of solid *N*-acetylglutamate, the mixture being magnetically stirred at 4°C. The supernatant was heated in a 65°C water bath until the temperature of the preparation reached 50°C, it was then kept in the water bath for an additional 1 min and gently swirled by hand. At the end of this time the suspension was transferred to an ice bath for 5 min, then centrifuged at 23300 ***g*** for 20 min and the pellet discarded. The supernatant was fractionated with saturated $(NH_4)_2SO_4$ solution at 4°C to give a 30–40%-satd.-$(NH_4)_2SO_4$ fraction, which was resuspended in Buffer B. In this buffer, enzyme activity was stable for at least 18 days at 4°C; in addition, it was found that glutamine synthetase activity, which was readily detectable in crude enzyme preparations, was completely absent from $(NH_4)_2SO_4$-treated preparations.

*Affinity chromatography*

Preliminary experiments with ATP linked in a number of different ways to agarose, indicated that *N*-acetylglutamate 5-phosphotransferase was strongly bound to ATP linked to agarose through the ribose hydroxy groups, via a six-carbon chain (AGATP, type 4).

A portion (25.0 ml) of heat- and $(NH_4)_2SO_4$-treated enzyme preparation was desalted through Sephadex G-50 with Buffer A. This was then applied to the ATP–agarose column and washed with 90.0 ml of Buffer A, followed by a pulse of ATP [3 ml of 0.05 M-ATP in 0.01 M-$KH_2PO_4$, 15% (v/v) glycerol, pH 7.5]. Fractions (5.0 ml) were collected and assayed for activity and protein concentration. Protein was determined by the method of Lowry *et al.* (1951). The elution profile of *N*-acetylglutamate 5-phosphotransferase demonstrated single activity and protein peaks that were coincident and maximal in fraction 25 immediately after the pulse of ATP. Fractions comprising peak activity were combined, and desalted on a Sephadex G-50 column (1.5 cm × 17 cm).

*DEAE-cellulose chromatography*

DEAE-cellulose was equilibrated with Buffer A in a column measuring 1.6 cm × 17 cm, and the enzyme preparation from the ATP–agarose column was applied. The column was washed with 75 ml of Buffer A and then proteins were eluted with a linear gradient comprised of 0–0.4 M-KCl in 0.01 M-potassium phosphate, containing 15% (v/v) glycerol.

Fractions containing significant phosphotransferase activity were pooled and were concentrated by using an Amicon ultrafiltration cell fitted with a PM10 membrane. The concentrated enzyme fraction was desalted through Sephadex G-50 and stored in Buffer A.

*Polyacrylamide-gel electrophoresis (with and without SDS), gel filtration and cross-linking*

Polyacrylamide-gel electrophoresis in 7% (w/v) gels was performed on all enzyme fractions; the running time was 95 min at 5 mA/gel. Gels were stained with Coomassie Brilliant Blue R-250, followed by destaining in acetic acid/methanol/water (3:2:35, by vol.) (Weber *et al.*, 1972). Fractions were diluted so that equal amounts (75 μg) of protein were applied to the gels. With homogeneous fractions, the amount of protein was decreased to 10 μg to avoid overloading.

A specific stain for $P_i$ was used to localize *N*-acetylglutamate 5-phosphotransferase activity (Sugino & Miyoshi, 1964). After electrophoresis, the

phosphate ions present in the gel were removed by cutting off the bottom portion of the gels. After incubation at 30°C in standard reaction mixture (consisting of 10mM-ATP, 10mM-$MgCl_2$, 100mM-N-acetylglutamate, 0.125M-Tris and 0.25M-hydroxylamine hydrochloride, pH 7.5) for 10min, the gels were removed, rinsed with deionized water and covered with an ammonium molybdate reagent consisting of 0.15M-$HClO_4$, 6mM-$(NH_4)_6Mo_7O_{24},4H_2O$ and 7mM-triethylamine hydrochloride (Sugino & Miyoshi, 1964). After about 10min a cream-coloured band appeared, indicating the position of the enzyme. The gels were stored in deionized water.

SDS/polyacrylamide-gel electrophoresis was performed as described by Laemmli (1970). A 3% (w/v) stacking gel, and 5% separating gel were used. Protein standards were chymotrypsinogen, cytochrome *c*, ovalbumin and bovine serum albumin.

Gel-filtration studies were performed with Sephadex G-150. The gel was equilibrated with Buffer A, which contained N-acetylglutamate, arginine, MgATP or no ligands. The enzyme was detected by assaying fractions for activity. Protein profiles were obtained as described by Lowry *et al.* (1951) after each fraction had been dialysed against 0.01M-$KH_2PO_4$. The protein standards used were yeast alcohol dehydrogenase, bovine serum albumin, ovalbumin and cytochrome *c*. The column void volume was determined in a separate run by using Blue Dextran 2000, since N-acetylglutamate 5-phosphotransferase was found to bind to Blue Dextran.

Cross-linking studies were performed by the method of Davies & Stark (1970) at 23°C for 3h. Dimethyl suberimidate was used at a final concentration of 1mg/ml, with protein at 75μg/ml. Cross-linking of yeast alcohol dehydrogenase was also performed as a control experiment (at 1mg of protein and 1mg of dimethyl suberimidate/ml). After cross-linking had been completed, the samples were denatured for 2h at 37°C in 1% SDS and 1% 2-mercaptoethanol. The total volume of each sample was adjusted to 200μl before electrophoresis at 8mA/tube (5% gels).

### *N-Terminal analysis*

The N-terminal analysis of purified enzyme was performed as described by Gros & Labouesse (1968). Purified N-acetylglutamate 5-phosphotransferase was dialysed against 0.01mM-$KH_2PO_4$, pH 7.5, which contained 0.1mM-EDTA and 1.0mM-2-mercaptoethanol. The dialysis residue (320μg of protein) was then freeze-dried and dissolved in 500μl of 8.33M-urea. To this was added 150μl of 0.4M-$NaH_2PO_4$, pH 8.2, 250μl of dimethylformamide and 100μl of 0.2M-dansyl chloride dissolved in acetonitrile. The starting pH was 9.5 and reaction was allowed to continue for 30min at room temperature in a conical Pyrex test tube capped with Parafilm. The final pH of the reaction mixture was 8.2. To this was added 6ml of 10% (w/v) trichloroacetic acid, which caused the formation of a fine precipitate. The solution was left for 1h at 4°C and the precipitate formed spun down in a clinical centrifuge at 2500rev./min ($r_{av.}$ 12cm). The supernatant was removed and the pellet washed twice with 2ml of 1M-HCl. The final pellet was resuspended in 2ml of 5.7M-HCl and transferred to a Teflon-capped vacuum hydrolysis tube (Pierce Chemical Co., Rockford, IL, U.S.A.). The tube was evacuated and placed in a Temp-Block (Pierce) at 110°C for 4h. The tubes were then taken out, and the HCl removed *in vacuo*. The residue was extracted with 20μl of acetone/acetic acid (3:2, v/v) and a 10μl sample was applied to a 10cm × 10cm polyamide thin-layer sheet (Pierce). The dansylated products were separated as described by Woods & Wang (1967). The identity of the dansylated products was determined by comparison with $R_F$ values of dansylated amino acid standards, run in one dimension and also two-dimensionally in the same solvent systems as the sample. Solvent system 1 was 1.5% formic acid and solvent system 2 was benzene/acetic acid (9:1, v/v).

### *Inhibitor studies*

The inhibitory effect of arginine on N-acetylglutamate 5-phosphotransferase activity was studied by using 50μl samples of purified enzyme (equivalent to 15μg of protein). Arginine was added to the samples to give the final desired concentration. Product inhibition by ADP, and alternate-substrate inhibition by adenosine 5′-[γ-thio]triphosphate and adenosine 5′-[α,β-methylene]triphosphate, were also studied in a similar manner.

A number of other compounds were tested as potential inhibitors; these included N-acetylaspartate, N-acetyl-γ-methoxyglutamate, glutamate, ornithine, N-acetylornithine, lysine, proline, γ-methoxyglutamate, norleucine and N-trifluoroacetylglutamate (synthesized as described by Allen & Jones, 1966). In all cases, three concentrations of potential substrate or inhibitor were investigated (1, 5 and 10mM) at two concentrations of N-acetylglutamate (10 and 50mM). The inhibitors were dissolved in Buffer A and the pH adjusted to 7.5. The concentration of ATP was kept constant at 10mM.

## **Results**

### *Enzyme-purification techniques*

Binding to ATP–agarose was found to be greater at low temperature (4°C) and in the presence of N-acetylglutamate (10mM), which yielded almost 100% of the total enzyme present bound to the column. Routinely, binding was performed in the

absence of *N*-acetylglutamate, since its presence appeared to lead to the eventual loss of the binding capacity of the ATP ligand. Running the agarose column in the absence of *N*-acetylglutamate increased the number of protein species that bound to the column, this being indicated by the appearance of multiple bands on polyacrylamide-gel electrophoresis. During chromatography on DEAE-cellulose, the major contaminating proteins were eluted with 0.15 M-KCl, whereas the peak fractions of *N*-acetylglutamate 5-phosphotransferase activity were eluted with 0.23 M-KCl. The enzyme peak had a small shoulder on the descending side; fractions comprising the shoulder were omitted, giving an enzyme preparation that appeared homogeneous and yielded one band in polyacrylamide-gel disc electrophoresis (Fig. 1). Similar analytical results were obtained when the purified enzyme was subjected to electrophoresis on continuous or discontinuous gradient gels. A summary of purification results is shown in Table 1. The steps used led to a 312-fold increase in the specific activity of the enzyme.

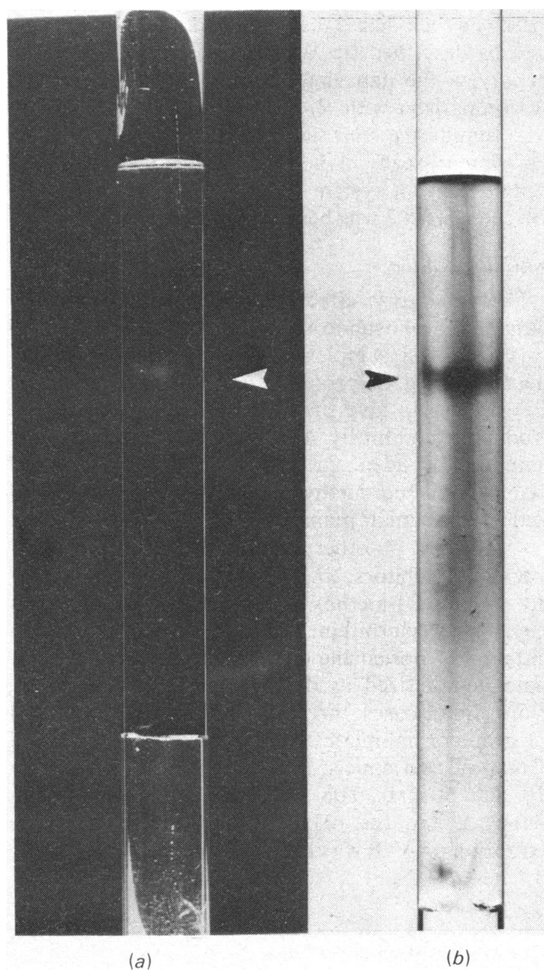



Fig. 1. *Analytical polyacrylamide-gel electrophoresis of purified N-acetylglutamate 5-phosphotransferase (10 μg of protein) run on 7% gels and stained with a specific activity stain (a) described in the Materials and methods section or Coomassie Brilliant Blue (b).*

*Initial velocity studies and arginine inhibition*

The initial velocity of the reaction was analysed by Cleland's (1963) procedure. Double-reciprocal plots of initial velocity versus [*N*-acetylglutamate] at a series of fixed concentrations of ATP (Fig. 2) resulted in a series of intersecting lines that deviated from linearity. A replot of these data on a Eadie–Hofstee plot demonstrated this non-linearity was real and indicated apparent negative co-operativity at high concentrations (>15 mM) of *N*-acetylglutamate. If [ATP] was kept constant, and instead [*N*-acetylglutamate] was varied at different concentrations of enzyme, then a family of curves was obtained, differing in slope but with similar maxima. When ATP was the varied substrate at a series of fixed concentrations of *N*-acetylglutamate, a series of intersecting lines was observed (Fig. 3). This pattern is consistent with a sequential reaction mechanism.

Replots of the *y*-intercepts versus the reciprocal of the fixed substrate concentration and shown as inserts in Figs. 2 and 3 give a single $K_m$ for ATP of $1.7 \times 10^{-3}$ M and two $K_m$ values for *N*-acetylglutamate of $1.9 \times 10^{-3}$ M and $6.2 \times 10^{-3}$ M.

When arginine was added to the incubation mixture, inhibition was seen (Figs. 4*a* and 4*b*). The inhibition seen with arginine resulted in sigmoid Michaelis plots when *N*-acetylglutamate was the varied substrate. The inhibition seen when ATP was the varied substrate resulted in normal Michaelis curves and was of a non-competitive nature, as shown by intersecting lines in a double-reciprocal plot (Fig. 5).

The inhibition seen with arginine when ATP was the substrate whose concentration was varied, was only apparent if [*N*-acetylglutamate] was kept at a low subsaturating value (i.e. 5 mM). When [*N*-acetylglutamate] was kept at 50 mM, no inhibition was seen.

The Hill plots produced from the inhibition data obtained when [*N*-acetylglutamate] was varied (Fig. 4) have slope or *h* values of 1.0 in the absence of arginine, and up to 2.0 with the greatest concentration of arginine used, indicative of the number of interacting sites on the enzyme (Koshland, 1970).

Product inhibition with ADP resulted in linear intersecting lines on reciprocal plots when [ATP] was varied and [*N*-acetylglutamate] was constant, or when [*N*-acetylglutamate] was varied and [ATP] was constant. These results are consistent

Table 1. *Purification of N-acetylglutamate 5-phosphotransferase by affinity chromatography and ion-exchange chromatography*

A unit of enzyme activity represents 1 μmol of product formed/min at 30°C.

| Fraction | 10 × Activity (units/ml) | Volume (ml) | Total activity (units) | Protein (mg/ml) | $10^3$ × Specific activity (units/mg of protein) | Recovery (%) | Purification (fold) |
|---|---|---|---|---|---|---|---|
| 1. Crude | 5.73 | 295 | 169 | 24.8 | 23.11 | (100) | (1) |
| 2. 30–40%$(NH_4)_2SO_4$ | 15.87 | 25 | 39.7 | 30.4 | 51.67 | 23.5 | 2.3 |
| 3. Heat-treated | 11.35 | 26 | 29.5 | 21.3 | 53.33 | 17.5 | 2.3 |
| 4. ATP–agarose (AGATP type 4) | 11.97 | 23.5 | 28.1 | 2.3 | 531.67 | 16.6 | 22.4 |
| 5. DEAE-cellulose DE 52 | 21.63 | 7 | 15.14 | 0.3 | 7210.00 | 9.0 | 312 |

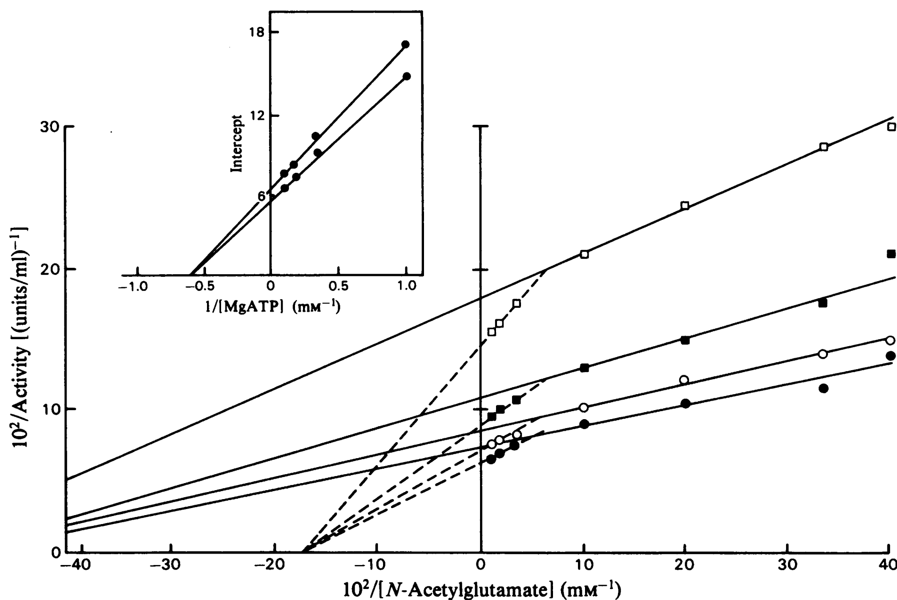



Fig. 2. *Initial-velocity analysis ([MgATP] fixed)*

A double-reciprocal plot at a series of fixed concentrations of MgATP is shown. MgATP concentration was kept at 1 mM (□), 3 mM (■), 6 mM (○) and 10 mM (●). The $K_m$ for MgATP is obtained by replotting the *y*-intercept values versus the reciprocal of the MgATP concentration (insert).

with non-competitive inhibition by ADP when [ATP] was varied and non-competitive inhibition when [*N*-acetylglutamate] was varied (Figs. 6*a* and 6*b*). Similar results were seen when adenosine 5′-[γ-thio]triphosphate was the inhibitor (results not shown). On the basis of the inhibition patterns seen with ADP, the suspected reaction mechanism of *N*-acetylglutamate 5-phosphotransferase is a random Bi Bi mechanism. Adenosine 5′[α,β-methylene]-triphosphate did not show any appreciable inhibition at any of the concentrations tested (1 and 10 mM, with [ATP] at 1 and 10 mM).

Various structural analogues of *N*-acetylglutamate were tested for their ability to act as substrates. Of the various compounds tried (*N*-acetylaspartic acid, glutamic acid, *N*-acetyl-γ-methoxyglutamic acid, ornithine, lysine, γ-methoxyglutamic acid, norleucine, proline and *N*-trifluoroacetylglutamic acid), only *N*-trifluoroacetylglutamic acid was able to act as a substrate, but was not as

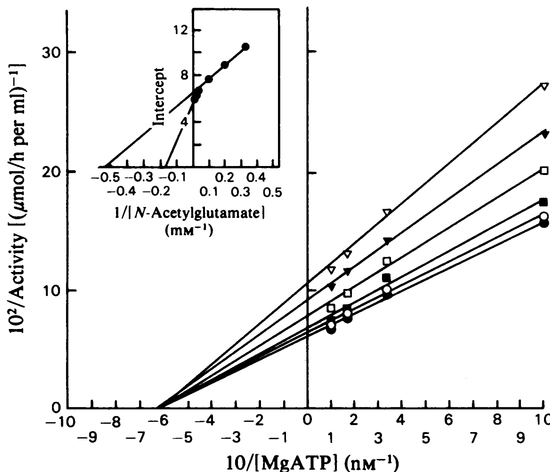



Fig. 3. *Initial-velocity analysis ([N-acetylglutamate] fixed)*

A double-reciprocal plot is shown for a series of fixed concentrations of *N*-acetylglutamate [3mM (▽), 5mM (▼), 10mM (□), 30mM (■), 60mM (○) and 100mM (●)]. The $K_m$ values for *N*-acetylglutamate were obtained by replotting the *y*-intercept values versus the reciprocal of the *N*-acetylglutamate concentration (insert).

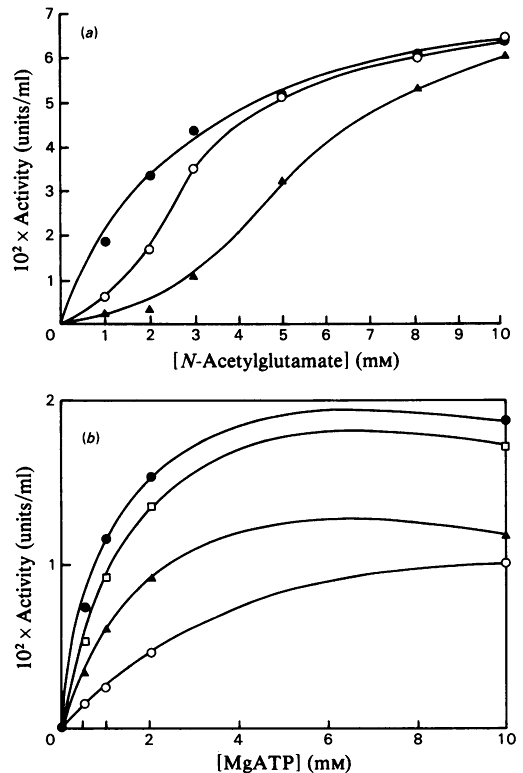



Fig. 4. *Inhibition of N-acetylglutamate 5-phosphotransferase by arginine*

(*a*) [MgATP] was constant at 10mM and arginine concentration kept at 0mM (●), 0.5mM (○) and 1.0mM (▲) with [*N*-acetylglutamate] varied over the range indicated. (*b*) [*N*-acetylglutamate] was constant at 10mM and [arginine] fixed at 0mM (●), 1.0mM (□), 1.25mM (▲) and 2.0mM (○), with [MgATP] varied over the concentrations indicated.

effective as *N*-acetylglutamate, showing only 76% the activity shown by an equivalent concentration of *N*-acetylglutamate.

*SDS/polyacrylamide-gel electrophoresis, gel filtration, cross-linking and N-terminal analysis of N-acetylglutamate 5-phosphotransferase*

SDS/polyacrylamide-gel electrophoresis of the enzyme (Laemmli, 1970) showed two major bands corresponding to 43000 and 53000mol.wt. (Fig. 7).

Gel filtration on Sephadex G-150 with buffer A demonstrated an enzyme activity peak corresponding to a mol.wt. of 93000 (Fig. 8*a*). The presence of various concentrations of ligands changed the elution profile. In the presence of 4.0mM-MgATP, no change in molecular weight was observed (Fig. 8*b*). The substrate *N*-acetylglutamate induced a shift in molecular weight up to a maximum of 190000, as can be seen in Figs. 9(*c*), 9(*d*) and 9(*e*). If arginine was present in the equilibration buffer, a similar shift in molecular weight to that observed for *N*-acetylglutamate was seen (Figs. 8*f* and 8*g*).

In spite of the use of a number of different micro-techniques, difficulty was experienced in accurately determining the very minute amounts of enzyme protein present in the samples obtained from gel-filtration studies. For this reason, we consider that the protein profiles represented in Fig. 8 only constitute an approximate guide for the protein concentrations in each fraction.

Cross-linking the purified enzyme demonstrated the production of three bands corresponding to higher molecular weight (85000, 115000 and 200000; Fig. 9).

*N*-Terminal analysis indicated the presence of two equally intense dansylated amino acids after t.l.c. These corresponded in $R_F$ to lysine and proline.

## Discussion

The increased binding of *N*-acetylglutamate 5-phosphotransferase to ATP–agarose in the presence of *N*-acetylglutamate could occur if *N*-acetylglutamate caused the active site of the enzyme to assume a conformation more favourable for the

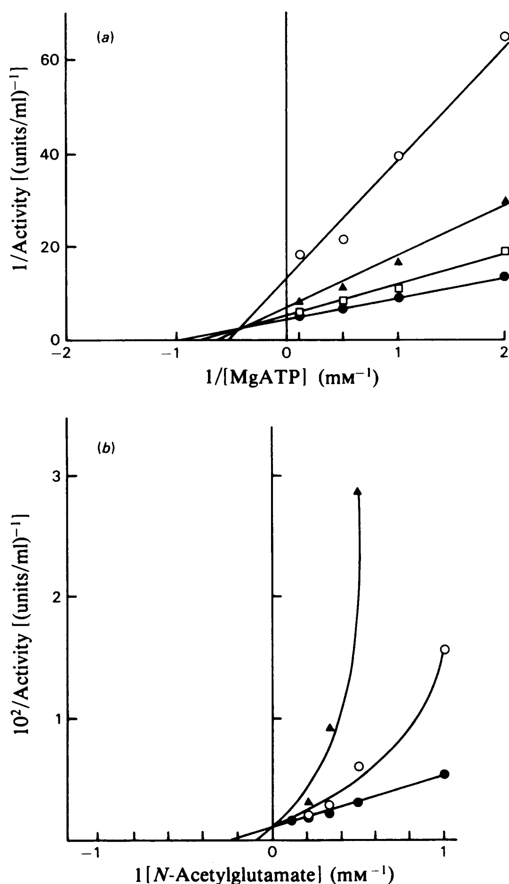



Fig. 5. *Double-reciprocal plots to determine type of inhibition by arginine with respect to ATP*

(*a*) Inhibition by arginine with respect to ATP, key as in Fig. 4(*b*); (*b*) inhibition by arginine with respect to *N*-acetylglutamate, key symbols are as for Fig. 4(*a*).

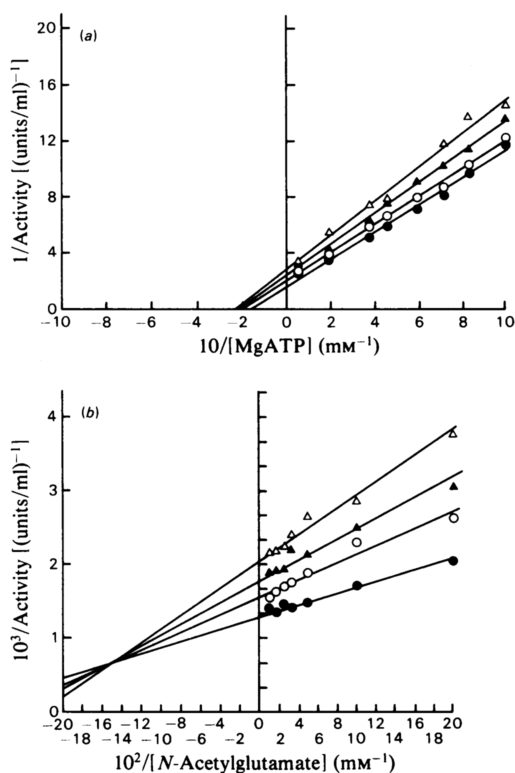



Fig. 6. *Double-reciprocal plots to determine type of inhibition by ADP with respect to ATP and N-acetylglutamate*

(*a*) [*N*-Acetylglutamate] was constant at 10mM with ADP fixed at 0mM (●), 1.0mM (○), 2.0mM (▲) and 3.0mM (△), while [MgATP] was varied over the range indicated; (*b*) [MgATP] constant at 5mM, with [ADP] fixed at 0.0mM (●), 1.0mM (○), 2.0mM (▲) and 3.0mM (△), while [*N*-acetylglutamate] was varied over the range indicated.

binding of ATP or created an enzyme form more easily bound to the affinity material. That *N*-acetylglutamate can cause structural changes, as shown by gel-filtration data, and can influence the activity of *N*-acetylglutamate 5-phosphotransferase by virtue of its negative-co-operativity effects, may be further evidence indicating altered substrate binding characteristics, as manifested by the increased binding of this enzyme to ATP–agarose in the presence of *N*-acetylglutamate.

The SDS/polyacrylamide-gel-electrophoretic evidence (Fig. 7) suggests that the enzyme consists of two subunits with mol.wts. of 53000 and 43000. Coupled with the gel-filtration data (Figs. 8*a* and 8*b*) and the results of *N*-terminal analysis, this suggests that the enzyme exists as a dimer with two non-identical subunits, and a combined mol.wt. of approx. 96000. As such, each dimer is composed of one subunit with mol.wt. 53000 and one subunit with mol.wt. 43000. An increase in the concentration of *N*-acetylglutamate brings about the self-association of these dimers to form a tetramer with a mol.wt. of approx. 190000 (Figs. 8*c*, 8*d* and 8*e*). Presumably the tetrameric form has altered kinetic properties (affinity for *N*-acetylglutamate such that the apparent negative co-operativity evident in Fig. 2 is observed). These enzyme forms were also made evident by the results of the cross-linking experiments (Fig. 9), where if we arbitrarily set the 53000-mol.wt. subunit an $\alpha$ designation and the 43000 subunit a $\beta$ designation, we can assign the cross-linked higher-molecular-weight bands $\alpha_2$, $\beta_2$

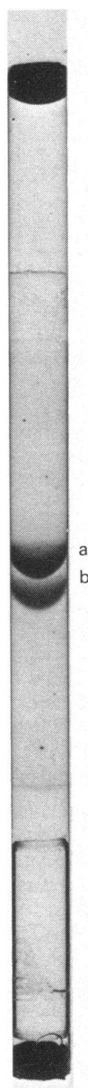



Fig. 7. *SDS/polyacrylamide-gel electrophoresis of purified N-acetylglutamate 5-phosphotransferase*
Bands a and b correspond to mol.wts. of 53000 and 43000. The electrophoresis was performed as described in the Materials and methods section.

and $\alpha_2\beta_2$ designations, corresponding to mol.wts. 85000, 115000 and 200000 respectively.

Haas & Leisinger (1975) have demonstrated ligand association–dissociation with $N$-acetylglutamate 5-phosphotransferase isolated from *Pseudo-*

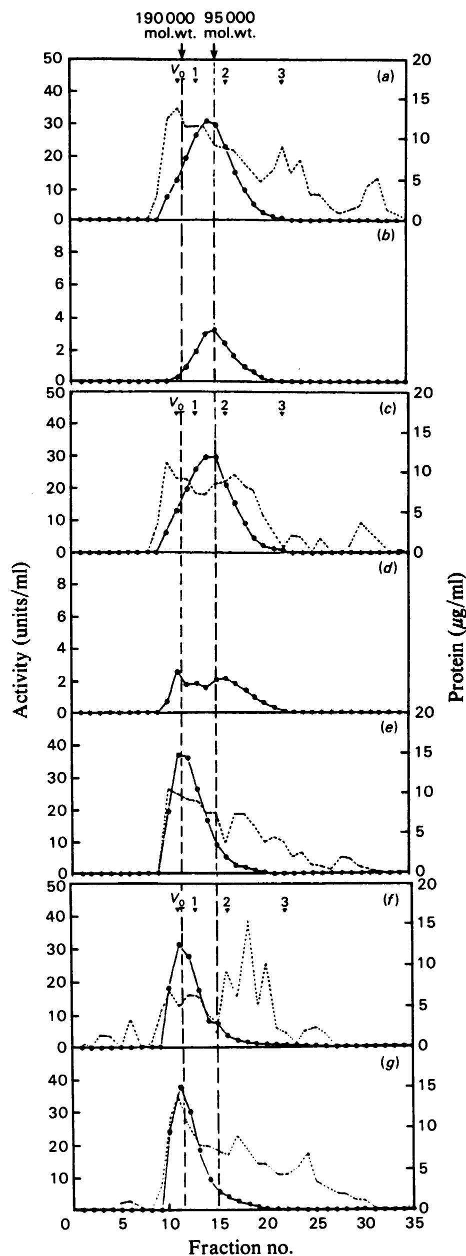



Fig. 8. *Gel filtration on Sephadex G-150 of N-acetylglutamate 5-phosphotransferase in the presence of various ligands*
(*a*) No ligands; (*b*) 4.0mM-MgATP; (*c*) 0.25mM-$N$-acetylglutamate; (*d*) 5.0mM-$N$-acetylglutamate; (*e*) 50.0mM-$N$-acetylglutamate; (*f*) 1.0mM-arginine and (*g*) 10.0mM-arginine. The above concentrations of ligands were dissolved in 0.01M-$KH_2PO_4$, 15% (v/v) glycerol, pH 7.5, and a column (1.5cm × 34cm) of Sephadex G-150 was equilibrated with the buffer before chromatography. Protein (----) was measured after dialysis as described in the text, and enzyme activity (●) was measured as described in the Materials and methods section. $V_0$ represents the void volume; the standards were yeast alcohol dehydrogenase (1), bovine serum albumin (2) and cytochrome *c* (3).

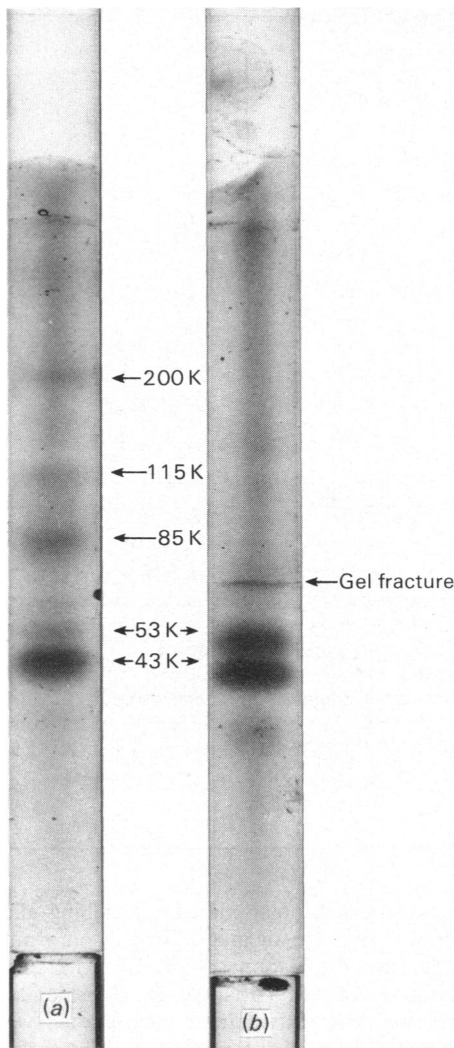
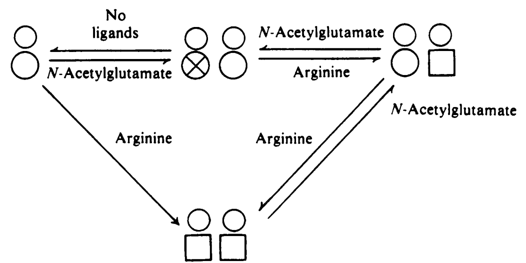



Fig. 9. *SDS/polyacrylamide-gel electrophoresis of cross-linked N-acetylglutamate 5-phosphotransferase*
Cross-linking was performed as described in the Materials and methods section at 23°C for 3h, with approx. 40μg of purified *N*-acetylglutamate 5-phosphotransferase and 0.5mg of dimethylsuberimidate. Gel (*a*) represents the cross-linked protein, whereas gel (*b*) represents an identically treated protein sample with the dimethylsuberimidate omitted. The molecular weights of the various protein bands are indicated (200K = 200000 mol.wt., etc.).

*monas aeruginosa.* In that system the enzyme exists in a higher-molecular-weight form in the absence of any ligands, and in the presence of arginine, a dissociation of subunits occurs. With the plant enzyme, arginine and *N*-acetylglutamate both appear to cause an association of dimers to tetrameric enzyme forms; however, the kinetic properties of these tetrameric forms are different. In the case of the arginine-induced tetramer, the enzyme is capable of displaying sigmoid kinetics when the concentration of *N*-acetylglutamate is varied. In the case of the *N*-acetylglutamate-induced tetramer, the enzyme has an altered affinity for *N*-acetylglutamate such that apparent negative co-operativity is displayed.




Scheme 1. *Postulated subunit interactions of N-acetylglutamate 5-phosphotransferase*
Circles represent active subunits that exist as a dimer in the absence of any ligands. Changes in enzyme structure due to the presence of various ligands are associated with changes in total enzyme molecular weight consistent with a dimer-to-tetramer formation. In the presence of elevated *N*-acetylglutamate concentrations, a tetramer with altered kinetic capacity is proposed and indicated by a circle with a cross. This tetramer displays negative co-operativity and may be associated with 'half-of-the-sites' activity. The squares represent inactive subunits. Only two subunits are shown to undergo sequential conformational changes consistent with the Hill interaction factor, *h*, of 2 in the presence of arginine.

It is apparent from the kinetic studies that increasing the concentration of *N*-acetylglutamate removes the inhibition exerted by arginine (Fig. 4*a*). This appears to indicate that *N*-acetylglutamate is the 'target substrate' of the allosteric inhibitor, arginine. The rate–concentration function for the inhibition pattern seen with arginine is sigmoidal when [*N*-acetylglutamate] is varied and [ATP] is fixed. The fact that arginine is essentially without effect on the $V_{max.}$ of the reaction (Fig. 5*b*) indicates that *N*-acetylglutamate 5-phosphotransferase falls into the 'K system' of allosteric enzymes (Monod *et al.*, 1965).

Taken collectively, our investigations allow the proposition of the model depicted in Scheme 1. In the model, *N*-acetylglutamate 5-phosphotransferase exists as a dimer. Increasing the concentration of *N*-acetylglutamate allows the formation of a tet-

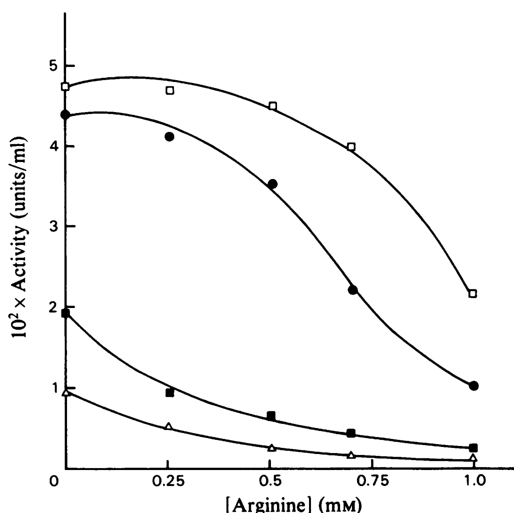



Fig. 10. *Initial velocity of N-acetylglutamate 5-phosphotransferase versus inhibitor concentration at a variety of fixed substrate concentrations*
The concentrations of ATP and $MgCl_2$ were 10mM, whereas that of *N*-acetylglutamate was fixed at: 0.5mM (△), 1.0mM (■), 3mM (●) and 4mM (□). One unit is 1 μmol of product formed/120min.

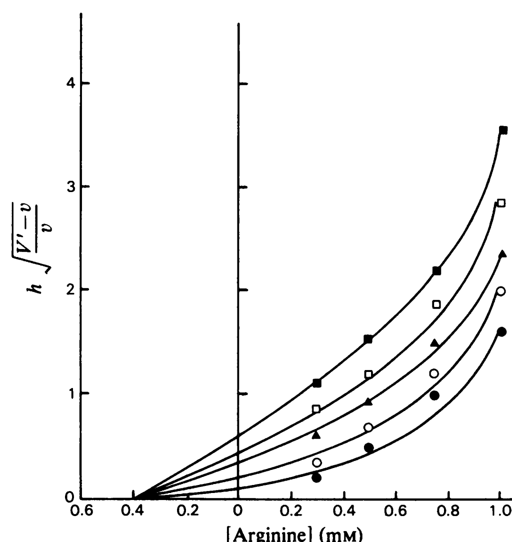



Fig. 11. *Replot of data from Fig. 4 (a)*
A plot of $h\sqrt{(V'-v)}/v$ versus the concentration of arginine is shown, where $h = 2$, $V$ is the uninhibited velocity at that concentration of *N*-acetylglutamate, and $v$ is the observed velocity in the presence of inhibitor. The concentration of MgATP was 10mM for all determinations. The concentration of *N*-acetylglutamate was: 1mM (■), 1.5mM (□), 2.0mM (▲), 2.5mM (○) and 3.0mM (●).

ramer, which has altered kinetic properties and displays negative co-operativity with respect to *N*-acetylglutamate. This enzyme form also has an increased capacity, either for steric reasons or by virtue of ATP-binding characteristics, to be adsorbed by ATP–agarose, as demonstrated by the increased binding to ATP–agarose in the presence of *N*-acetylglutamate. Arginine induces a molecular-weight change similar in magnitude to that induced by *N*-acetylglutamate, but the resultant dimer of dimers is catalytically inactive.

No data have been obtained so far to suggest why the enzyme should consist of two different types of subunit. It would seem likely that the various types of substrates and inhibitors utilized in the present study are bound to different subunits (either α or β). Exactly where such sites are situated and which substrates bind to them has not been definitely determined. It would seem likely, however, that the site (or sites) utilized by *N*-acetylglutamate and arginine are distinct from that utilized by ATP.

When the data of Fig. 4(*a*) are replotted as a function of the inhibitor concentration, a series of curves is obtained that increase in sigmoidicity as the concentration of *N*-acetylglutamate increases (Fig. 10). This pattern is consistent with a model involving conformational changes, together with the associated changes in affinity of the enzyme for either the substrate or inhibitor (Monod *et al.*, 1965).

The two most popular models describing allosteric proteins and associated with conformational changes are the Symmetry model (Monod *et al.*, 1965) and the Sequential model (Koshland *et al.*, 1966). On first examination the Symmetry model does not predict all the characteristics of *N*-acetylglutamate 5-phosphotransferase, since this model does not adequately describe negative co-operativity, owing to its more stringent assumption of 'all or none' conformational transitions (Hammes & Wu, 1974). Further evidence ruling out the Symmetry model is the finding that a replot of the data from Fig. 4(*a*) by the method of Segel (1975) and shown in Fig. 11 yields a family of curves that are not linear. If the evaluation of the data according to the Symmetry model were correct, a series of straight lines intersecting at a common point and yielding the dissociation constant of the inhibitor for the enzyme in the T-state should have been obtained (Segel, 1975).

A better explanation for the data of Fig. 4(*a*) would be a sequential change in enzyme subunit conformation. Under such conditions, the binding of inhibitor to one site does not necessarily result in an identical change in the affinities of all the remaining vacant sites; the net result is a variable dissociation

constant for both the substrate and inhibitor that depends on the relative concentrations of these ligands. As a result, non-linear curves such as those seen in Fig. 11 are expected.

The results of studies *in vitro* performed on purified samples of the enzyme *N*-acetylglutamate 5-phosphotransferase appear to indicate an important regulatory role for the enzyme *in vivo*. Apart from being the first truly 'committed' enzyme of the pathway of ornithine synthesis as it exists in plants (McKay & Shargool, 1977), the enzyme appears to be subject to activation by *N*-acetylglutamate and to inhibition by arginine. Thus this strategically placed enzyme can significantly influence the rate of the biosynthesis of arginine as well as ornithine.

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