- Tsukada, Y., Hirano, S., Nagata, Y. & Matsutani, T. (1960). In *Inhibition in the Nervous System and Gamma-Aminobutyric Acid*, p. 163. Ed. by Roberts, E. London: Pergamon Press Ltd.
- Van Slyke, D. D. & Folch, J. (1940). *J. biol. Chem.* **136**, 509.
- Velick, S. F. & Vavra, J. (1962). *J. biol. Chem.* **237**, 2109.
- Vrba, R., Gaitonde, M. K. & Richter, D. (1962). *J. Neurochem.* **9**, 465.
- Waelisch, H. (1949). *Lancet*, ii, 1.
- Waelisch, H. & Lajtha, A. (1961). *Physiol. Rev.* **41**, 709.
- Weinman, E. O., Strisower, E. H. & Chaikoff, I. L. (1957). *Physiol. Rev.* **37**, 252.
- Wilson, W. E., Hill, R. J. & Koeppe, R. E. (1959). *J. biol. Chem.* **234**, 347.
- Winzler, R. J., Moldave, K., Rafelson, M. E., jun. & Pearson, H. E. (1952). *J. biol. Chem.* **199**, 485.

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Properties of Crystalline L-Aspartate 4-Carboxy-Lyase from *Achromobacter* sp.

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L-Aspartate 4-carboxy-lyase (EC 4.1.1.12) has been shown to be present in a number of micro-organisms (for review, see Braunstein, 1960). The enzyme, which is unusual in its strong stimulation by α -oxo acids, was partially purified by Nishimura, Manning & Meister (1962) and was shown to contain firmly bound pyridoxal 5'-phosphate; it has subsequently been crystallized from *Achromobacter* sp. (Wilson, 1963) and has been extensively purified from *Alcaligenes faecalis* (Meister, Nishimura & Novogradsky, 1962).

It is the main purpose of the present paper to describe some of the properties of the L-aspartate 4-carboxy-lyase of *Achromobacter* sp. and, in particular, to discuss the mode of binding of pyridoxal 5'-phosphate to the protein and to L-aspartate. A preliminary report of part of this work has been presented to The Biochemical Society (Wilson & Kornberg, 1963).

METHODS AND MATERIALS

Preparation of L-aspartate 4-carboxy-lyase. The enzyme was purified by the procedure of Wilson (1963) except that the conditions of crystallization were modified to obtain more uniform crystals. The first crop of crystals was obtained by dialysis of the carboxymethylcellulose-treated extract ('Step V') against 0.2M-sodium acetate, pH 7.0, instead of against 0.1M-buffer as previously described. It is important that the dialysis be performed without stirring of the buffer. The enzyme was recrystallized by the same procedure. The resulting crystals (Fig. 1) were regular hexagonal plates that became broken and less regular with time (the photographs shown in Fig. 1 were taken after the crystals had been stored for 3 days).

Two preparations of enzyme were used in most of the experiments described below. One had an initial specific activity of 107 units/mg. of protein and contained 1 mole of

pyridoxal 5'-phosphate/120 000 g. of protein, and was used in the spectrophotometric experiments; the other had an initial specific activity of 90 units/mg. of protein and contained 1 mole of pyridoxal 5'-phosphate/60 000 g. of protein; this preparation was used in the ultracentrifugal studies (see Appendix and Fig. 2) and in the remaining experiments.

The enzyme was stable when stored in concentrated solution at pH 7 and 4°. Activity was lost very rapidly in dilute solution or at pH 5.0; it is recommended that dilution before assay be made in 0.5% bovine serum albumin.

A solution of the second preparation of enzyme in 0.5M-sodium acetate, pH 7.0, containing 8.8 mg. of protein/ml., lost less than 15% of its activity, when assayed in the presence of pyruvate, after storage for 2½ months. However, its activity assayed in the absence of pyruvate doubled after this storage. This stored enzyme, which was therefore stimulated less by pyruvate than was freshly prepared enzyme, is referred to below as 'aged' enzyme.

Assay methods. Method A. L-Aspartate 4-carboxy-lyase was assayed manometrically at 30°. Evolution of CO₂ was measured for 30 min. after the addition of L-aspartate, and was linear with time and with enzyme concentration. The main compartments of Warburg flasks contained, in a final volume of 1.8 ml., 200 µmoles of sodium acetate (pH 5.0), 0.5 µmole of sodium pyruvate, and enzyme (2–2000 µg. of protein). L-Aspartate (20 µmoles) was added from the side arm after equilibration for 15 min. One unit of enzyme is defined as that quantity which catalyses the evolution of 1 µmole of CO₂/min. under these conditions.

Method B. When lower substrate concentrations were required, the activity of the enzyme was measured by assay of the radioactivity of ¹⁴CO₂ from the decarboxylation of L-[U-¹⁴C]aspartate. The complete system contained, in the main compartments of double-armed Warburg manometer cups, 200 µmoles of sodium acetate (pH 5.0), 0.5 µmole of sodium pyruvate, 0.176 µg. of purified L-aspartate 4-carboxy-lyase, and water to bring the reaction volume to 2.0 ml. after the addition of the contents of the first side arm. The centre wells contained 0.2 ml. of 2N-KOH. L-[U-¹⁴C]aspartate (0.05–0.20 µmole) was added from the

first side arm after equilibration for 15 min. at 30°. The reaction was terminated after various times by the addition of 0.1 ml. of 5N-H₂SO₄ from the second side arm. The cups were detached 15 min. later and the contents of the centre wells were mixed with 189 μ moles of Na₂CO₃. The carbonate was precipitated as BaCO₃ with BaCl₂; samples of the precipitated BaCO₃ (0.4 vol.) were plated by filtration (Sakami, 1955) and their radioactivities assayed with a mica end-window β -counter tube (G.E.C., type 2B2). The observed radioactivity (3000–9000 counts total) was corrected for background radiation and self-absorption to infinite thinness. In each series of determinations, the observed radioactivity was converted into the quantities of L-aspartate decarboxylated by determining the total radioactivity contained in C-4 of 0.05 μ mole of [¹⁴C]aspartate, by using the same procedure and an excess (1.76 μ g.) of enzyme. The velocities of decarboxylation were linear with enzyme concentration and with time for the first 10 min. even with the lowest L-aspartate concentration used.

Spectrophotometric studies. Spectra were measured with an Optica Recording Grating Spectrophotometer, CF4, in 1.5 ml. silica micro-cells of 1 cm. light-path.

Reduction of the enzyme with sodium borohydride. A fresh aqueous solution (0.1 ml.) of sodium borohydride (10 mg./ml. or 2 mg./ml.) was added to 1 ml. of enzyme (1.7 or 3 mg. of protein) in 0.1 M-sodium acetate, pH 5.0, in a centrifuge

tube; the tube was immediately centrifuged for 30 min. at 2000 g and at 0° to reduce foaming (Hughes, Jenkins & Fischer, 1962). The mixture was then dialysed overnight against 0.1 M-sodium acetate, pH 5.0.

Determination of protein. Protein concentrations were measured by the method of Lowry, Rosebrough, Farr & Randall (1951). A standard curve was derived from the assay of crystalline bovine serum albumin (Nutritional Biochemicals Corp., Cleveland 28, Ohio, U.S.A.).

Radioactive materials. L-[U-¹⁴C]Aspartic acid, purchased from The Radiochemical Centre, Amersham, Bucks., and containing about 22 μ C/ μ mole, was dissolved in water and purified by chromatography on Whatman no. 3 paper in the butanol-propionic acid-water solvent of Calvin & Benson (1949). The radioactive band on the dried chromatograms, located by radioautography, was eluted with water. The [¹⁴C]aspartate was diluted about ninefold with carrier potassium L-aspartate and the concentration adjusted to 1 mM after standardization by the quantitative ninhydrin method of Moore & Stein (1954).

Chemicals. DL-threo- β -Hydroxyaspartic acid, prepared as described by Kornguth & Sallach (1960), was a gift from Dr J. G. Morris. DL-erythro- β -Hydroxyaspartic acid was purchased from the California Corp. for Biochemical Research (Los Angeles, U.S.A.). Other amino acids, sodium borohydride, bovine serum albumin, glyoxal, glyoxylic acid

Fig. 1. Crystals of L-aspartate 4-carboxy-lyase viewed by dark-ground illumination. (a) Magnification $\times 176$; (b) magnification $\times 352$.

Fig. 2. Sedimentation pattern of crystalline L-aspartate 4-carboxy-lyase. The enzyme was dissolved in 0.5 M-sodium acetate, pH 7.0, at 7.8 mg./ml. Sedimentation was from left to right at phase-plate angle 70° and rotor temperature 18°. (Photographed by Mr P. H. Lloyd, Department of Biochemistry, University of Oxford; see the Appendix.)

and isoniazid were purchased from British Drug Houses Ltd. (Poole, Dorset); oxaloacetic acid, sodium pyruvate and α -oxoglutaric acid were from C. F. Boehringer und Soehne (Mannheim, Germany) and pyridoxal 5'-phosphate was from F. Hoffmann-La Roche and Co. (Basel, Switzerland). All other reagents used were of the highest purity commercially available.

RESULTS

Effect of substrate and substrate analogues on enzymic activity. The Michaelis constant, K_m , for L-aspartate was found to be $80 \mu\text{M}$ by the method of Lineweaver & Burk (1934).

When each of the substrate analogues listed in Table 1 was tested as a substrate at a final concentration of 10 mM by assay method A, none was decarboxylated. When the same compounds were tested for inhibition (Table 1), three substituted aspartic acids and two C_4 dicarboxylic acids inhibited by 40 % or more, whereas other compounds had weaker or negligible inhibitory action. The specificity of the enzyme for dicarboxylic acid inhibitors is similar to that of L-aspartate-2-oxoglutarate aminotransferase (Jenkins, Yphantis & Sizer, 1959): both enzymes are inhibited by succinate but not by malonate, and by maleate but not by fumarate. The interaction of both enzymes with maleate probably explains their protection by this buffer against heat-inactivation during purification.

Jenkins (1961a) found that *erythro*- β -hydroxyaspartate and *threo*- β -hydroxyaspartate react

Table 1. Inhibition of L-aspartate 4-carboxy-lyase by analogues of L-aspartate

L-Aspartate 4-carboxy-lyase was assayed by assay method B (see the text) but with $0.088 \mu\text{g}$. of enzyme/cup. The first side arm contained $0.1 \mu\text{mole}$ of $[\text{U-}^{14}\text{C}]$ aspartate and $10 \mu\text{moles}$ of the analogue to be tested. Inhibition is expressed as the rate observed with L-aspartate and added compound as a percentage of the rate observed with L-aspartate alone. In the absence of inhibitor, $2.64 \mu\text{moles}$ of L-aspartate were decarboxylated/min. at 30° .

Added compound	Rate (% of uninhibited rate)
None	100
Fumarate	100
Malonate	96
L-Threonine	95
L-Glutamate	94
L-Serine	92
L-Malate	90
β -Alanine	88
(+)-Tartrate	82
α -L-Alanine	79
DL- β -Methylaspartate	60
Maleate	59
Succinate	32
DL- <i>erythro</i> - β -Hydroxyaspartate	18
DL- <i>threo</i> - β -Hydroxyaspartate	2

reversibly with L-aspartate-2-oxoglutarate aminotransferase. Fig. 3 shows that DL-*threo*- β -hydroxyaspartate and DL-*erythro*- β -hydroxyaspartate are competitive inhibitors also of L-aspartate 4-carboxy-lyase, with K_i values of $11 \mu\text{M}$ and $320 \mu\text{M}$ respectively. When the enzyme was preincubated with the inhibitor before the addition of the L-aspartate, the rates in the presence of the inhibitor were linear under the conditions of assay method B; under the conditions of assay method A, where larger amounts of L-aspartate were added, they were linear only after a 5 min. lag.

Activation by keto compounds. Table 2 shows the activation of two different preparations of the enzyme by a variety of keto compounds. Glyoxal as well as 2-oxo acids activated the enzyme. The rates of decarboxylation in the absence of added keto compounds were twice as high with

Fig. 3. Effect of L-aspartate concentration and inhibitors on the rate of action of L-aspartate 4-carboxy-lyase. The enzyme was assayed, in the absence of inhibitors (O), and after preincubation for 15 min. with either 1 mM DL-*erythro*- β -hydroxyaspartate (∇) or 0.02 mM DL-*threo*- β -hydroxyaspartate (Δ), by assay method B (see the text). Rates at saturating substrate concentrations ($\bullet$, $\blacktriangledown$, $\blacktriangle$) were obtained by assay method A (see the text), with $8.8 \mu\text{g}$. of enzyme/flask.

'aged' enzyme as with freshly prepared enzyme, but this activity was almost completely eliminated in both preparations by the addition of L-aspartate in concentrations much higher than those used in assay method *A*. In the presence of 0.1M-L-aspartate, the K_m for pyruvate was found to be 6.25 μ M by the method of Lineweaver & Burk (1934). Under these conditions, only a very low rate of aspartate decarboxylation occurred in the absence of pyruvate.

Inhibition by carbonyl reagents. Table 3 shows the concentrations of carbonyl reagents that caused partial inhibition of an 'aged' preparation of enzyme (which did not require added pyruvate for activity). The addition of potassium cyanide, hydroxylamine or phenylhydrazine lowered the rates of aspartate decarboxylation, but these rates were linear with time and were not affected by the subsequent addition of pyruvate. However, the degree of inhibition produced by relatively high

concentrations of semicarbazide or isoniazid increased with time; this inhibition was partially reversed immediately after the addition of pyruvate to give linear rates (Table 3 and Fig. 4). The observations that, after preincubation of the enzyme with semicarbazide for various times, the initial rates of reaction and the time required for complete inhibition were the same suggest that semicarbazide cannot react with the enzyme alone but only with the enzyme-substrate complex. Support for this hypothesis was derived from the finding that semicarbazide reacted with the complex formed between the enzyme and an aspartate analogue. When the enzyme was preincubated with 1 mM-semicarbazide and 1 mM-DL-erythro- β -hydroxyaspartate, the inhibited rate was only 25 % of the

Table 2. Activation of L-aspartate 4-carboxy-lyase by keto compounds

Assay method *A* (see the text) was used except that the 0.5 μ mole of pyruvate was replaced by an equal amount of other keto compounds, as listed. In the assays marked with an asterisk, the final concentration of L-aspartate was 0.1M.

Keto compound	Specific activity (units/mg. of protein)	
	Fresh enzyme	'Aged' enzyme
None	25	52
Pyruvate	70	79
α -Oxoglutarate	64	80
Oxaloacetate	64	76
Glyoxylate	47	49
Glyoxal	60	73
Acetaldehyde	37	58
None*	0	5
Pyruvate*	70	76

Fig. 4. Effect of semicarbazide, isoniazid and pyruvate on the rate of action of L-aspartate 4-carboxy-lyase. 'Aged' enzyme (8.8 μ g.) was assayed by method *A* (see the text) except that, in curves 4-9, pyruvate (placed in the second side arm, instead of in the main compartment) was added where indicated by arrows. In the experiments of curves 2, 4, 6 and 8 the flasks contained 1 mM-semicarbazide in the main compartment; in those of curves 3, 5, 7 and 9 the flasks contained 10 mM-isoniazid in the main compartments. The contents of the main compartments were preincubated for 15 min. (curves 1-5), 30 min. (curves 6 and 7) or 45 min. (curves 8 and 9) before the addition of L-aspartate.

Table 3. Inhibition of L-aspartate 4-carboxy-lyase by carbonyl reagents

Assay method *A* (see the text) was used except that the pyruvate was placed in the second side arm and added 20 min. after the L-aspartate. 'Aged' enzyme and the carbonyl reagents were placed in the main compartments of the Warburg cups. Percentage inhibition is expressed as the rate without carbonyl reagent minus the rate with carbonyl reagent, as a percentage of the rate without carbonyl reagent. Assays marked with an asterisk showed progressive inhibition (up to 85 % between 15 and 20 min.).

Carbonyl reagent added	Assay before addition of pyruvate		Assay after addition of pyruvate	
	Rate (units/mg.)	Inhibition (%)	Rate (units/mg.)	Inhibition (%)
None	59	—	67	—
0.1 mM-KCN	43	28	43	36
0.1 mM-Hydroxylamine	18	70	20	70
0.01 mM-Phenylhydrazine	32	45	36	45
1.0 mM-Semicarbazide	*	*	48	29
10.0 mM-Isoniazid	*	*	34	49

mean between the rate observed with enzyme preincubated with semicarbazide and that observed with enzyme preincubated with DL-erythro- β -hydroxyaspartate.

The reversal of the semicarbazide inhibition was not specific for pyruvate. All of the keto compounds listed in Table 2 restored the rate of decarboxylation to 70–100 % of that observed in the absence of semicarbazide, except acetaldehyde, which was ineffective, and glyoxylate, which caused an immediate activation, followed by a second, progressive inhibition.

The effects obtained with isoniazid differed from those with semicarbazide; the longer the enzyme was preincubated with isoniazid, the smaller was the amount of carbon dioxide evolved in the absence of pyruvate and the less complete was the restoration of activity produced by pyruvate (Fig. 4). These results suggest that there are two types of inhibition by isoniazid, one reversible and independent of the time of preincubation, and the other irreversible and dependent on the period of preincubation. The possibility of two modes of inhibition by isoniazid has also been suggested by Davison (1956) and by Jenkins, Orlowski &Sizer (1959).

Fig. 5 shows the spectra of the enzyme and of its derivatives with cyanide and hydroxylamine. The formation of the hydroxylamine derivative was immediate; dialysis restored both the 360 m μ peak and full enzymic activity. The spectrum of this derivative is similar to that reported for the hydroxylamine derivative of L-homoserine hydroxylase (deaminating) (Matsuo & Greenberg, 1958) and is different from the hydroxylamine derivative

Fig. 5. Effect of carbonyl reagents on the spectrum of L-aspartate 4-carboxy-lyase. Curve 1: 1.5 mg. of enzyme in 1.0 ml. of 0.1M-sodium acetate, pH 5.0; curve 2: enzyme (as for curve 1) 15 min. after the addition of 0.1 ml. of 0.1M-hydroxylamine hydrochloride; curve 3: enzyme (as for curve 1) 45 min. after the addition of 0.1 ml. of 0.1M-KCN.

of free pyridoxal 5'-phosphate. The formation of the cyanide derivative was progressive over 30 min.; dialysis caused no change in the spectrum and left the dialysed enzyme with only 10 % of the activity observed before the cyanide treatment. The cyanohydrin of free pyridoxal 5'-phosphate has the same extinction maximum (330 m μ) as the cyanide derivative of the enzyme. However, it is likely that this interaction involves more than the formation of a simple cyanohydrin since, in that case, the cyanide would be expected to be removed by dialysis. The cyanide derivatives of L-homoserine hydro-lyase (deaminating) (Matsuo & Greenberg, 1958) and L-aspartate-2-oxoglutarate aminotransferase (Sizer & Jenkins, 1962) have different extinction maxima, at 360 and 339 m μ respectively.

Effect of pH on the enzyme. The pH for optimum enzymic activity was found to extend over a wide range from 3.5 to 6.0 (Fig. 6). It was also observed that there was no appreciable change in the spectrum of the enzyme between pH 3 and 8. Both observations indicate that the protein-bound pyridoxal 5'-phosphate does not undergo any ionization in this pH range. L-Aspartate 4-carboxy-lyase was strongly inhibited by phosphate buffers at pH values greater than 5.0.

Other inhibitors. L-Aspartate 4-carboxy-lyase was tested for stimulation or inhibition by a variety of metal salts, chelating agents and sulphydryl reagents at 0.1 mM concentrations: zinc sulphate,

Fig. 6. Effect of pH on the activity of L-aspartate 4-carboxy-lyase. Assay method A (see the text) was used except that 200 μ moles of glycine-HCl buffer (▲), sodium acetate buffer (○) or histidine buffer (▼), at the pH indicated, was substituted for the acetate buffer, pH 5.0; 8.8 μ g. of enzyme was used for each determination. The second side arm of each Warburg cup contained 0.15 ml. of 5N-H₂SO₄, which was added 15 min. after the addition of the L-aspartate. CO₂ evolution was measured 15 min. after the addition of the acid and corrected for a control in which the acid was added before the aspartate.

cupric sulphate, ferrous sulphate, ferric chloride, $\alpha\alpha'$ -bipyridyl, dithizone, *o*-phenanthroline, *p*-hydroxymercuribenzoate, iodoacetate and *N*-ethylmaleimide were tested. None inhibited or stimulated more than 10% under the conditions of assay method A and with the enzyme preincubated with the substances to be tested for 15 min. at 30° before assay.

Binding of pyridoxal 5'-phosphate. Pyridoxal 5'-phosphate has been shown to be present in L-aspartate 4-carboxy-lyase (Wilson, 1963) by microbiological assay of the acid-hydrolysed enzyme and by colorimetric determination with the phenylhydrazine assay of Wada & Snell (1962). Variable amounts of pyridoxal 5'-phosphate have been found by the latter assay method in different preparations of the crystalline enzyme. However, the activity of different preparations was not proportional to the total amount of pyridoxal 5'-phosphate present; the two most active preparations of the enzyme contained only one-half of the pyridoxal 5'-phosphate present in two other preparations.

Treatment of the enzyme at pH 5.0 with sodium borohydride resulted in a spectral shift from 360 to 325 m μ , which was not reversed by dialysis (Fig. 7a) and a loss of activity (Table 4). When the dialysed protein was precipitated by acid, neither material absorbing at 295 m μ nor material that reacted with phenylhydrazine was detected in the supernatant solution. Hence, neither pyridoxal 5'-phosphate nor its reduction product, pyridoxol 5'-phosphate, was released by acid from the enzyme treated with sodium borohydride, although acidification has been shown to release pyridoxal 5'-phosphate from the untreated enzyme (Wilson, 1963). These observations indicate that, by treatment with sodium borohydride, all of the pyridoxal 5-phosphate was stably bound to the enzyme, probably in a reduced form.

The protein that was precipitated by acid after sodium borohydride treatment was hydrolysed in 6*N*-hydrochloric acid for 16 hr. at 100°. The pyridoxal 5'-phosphate derivative of bovine serum albumin was prepared, treated with sodium borohydride, and hydrolysed under conditions reported to yield ϵ -pyridoxyl-lysine (Dempsey & Christensen, 1962). Both protein hydrolysates were chromatographed on Whatman no. 3 paper in the solvent system of Dempsey & Christensen (1962). Both hydrolysates showed blue-white fluorescent spots, with R_f 0.39, when examined in ultraviolet light; this fluorescence was quenched by ammonia vapour. These results indicate that the material, present in the hydrolysate of the sodium borohydride-treated enzyme, was indeed ϵ -pyridoxyl-lysine.

The addition of 100 μ moles of L-aspartate to the enzyme caused an immediate shift of the spectral

peak from 360 to 325 m μ . This shift was not reversed after 1 hr., but it was immediately reversed on the addition of 5 μ moles of sodium pyruvate. With such high concentrations of L-aspartate, the enzyme is almost inactive in the absence of pyruvate (Table 2). However, if lower concentrations of aspartate are used, the enzyme is active though no pyruvate is added; under these conditions, the relatively large quantities of enzyme necessary for spectral measurements catalysed the decarboxylation of all the substrate added before spectral changes could be recorded. A similar spectral shift with L-aspartate and reversal by

Fig. 7. Effect of sodium borohydride and DL-erythro- β -hydroxyaspartate on the spectrum of L-aspartate 4-carboxy-lyase. (a) Curve 1: 1.7 mg. of enzyme in 1.0 ml. of 0.1 M-sodium acetate, pH 5.0; curve 2: enzyme (as for curve 1) after treatment with 1 mg. of sodium borohydride and dialysis. (b) Curve 1: 1.7 mg. of enzyme in 1.0 ml. of 0.1 M-sodium acetate, pH 5.0; curve 2: enzyme (as for curve 1) 30 min. after the addition of 0.01 ml. of 0.1 M-DL-erythro- β -hydroxyaspartate; curve 3: enzyme (as for curve 2) 30 min. after treatment with 1 mg. of sodium borohydride; curve 4: enzyme (as for curve 3) after dialysis.

Table 4. *Effect of sodium borohydride and DL-erythro- β -hydroxyaspartate on the activity of L-aspartate 4-carboxy-lyase*

The assays were performed by method A (see the text) on enzyme that had been treated (Fig. 7b) with 1 μ mole of DL-erythro- β -hydroxyaspartate and 1 mg. of sodium borohydride (Expt. 1) or on enzyme (3 mg. of protein in 1.0 ml. of 0.1 M-sodium acetate, pH 5.0) that was treated in the same way with 0.2 mg. of sodium borohydride (Expt. 2). All assays were done after dialysis.

Treatment	Expt. 1		Expt. 2	
	Specific activity (units/mg. of protein)	Pyridoxal 5'-phosphate content (μ m-moles/mg. of protein)	Specific activity (units/mg. of protein)	Pyridoxal 5'-phosphate content (μ m-moles/mg. of protein)
None	108	8.3	73	6.55
Sodium borohydride	0	0	2	< 1.6
DL-erythro- β -Hydroxyaspartate	81	—	—	—
DL-erythro- β -Hydroxyaspartate + sodium borohydride	21	6.25	40	6.1

pyruvate have been observed by Meister *et al.* (1962).

When only 1 μ mole of the competitive inhibitor, DL-erythro- β -hydroxyaspartate, was added to the enzyme, the extinction at 360 $m\mu$ decreased at a rapid initial rate: half of the final decrease occurred within the first 2 min. and no further decrease occurred after 30 min. The spectrum of the enzyme thus treated (Fig. 7b: curve 2) had a maximum at 325 $m\mu$ with a small shoulder at 360 $m\mu$. No change in this spectrum was observed on the subsequent addition of 2 μ moles of sodium pyruvate. Treatment of this solution with sodium borohydride resulted in the removal of the shoulder at 360 $m\mu$ and an increase in the extinction at 325 $m\mu$ (Fig. 7b: curve 3). Dialysis of the enzyme thus treated resulted in the formation of a broad peak with a maximum at 350 $m\mu$ (Fig. 7b: curve 4) and a partial restoration of activity (Table 4). When this enzyme was assayed by the phenylhydrazine method, it was found to contain 1 mole of pyridoxal 5'-phosphate/160 000 g. of protein. The untreated enzyme contained 1 mole of pyridoxal 5'-phosphate/120 000 g. of protein, which shows that only a small proportion of the pyridoxal 5'-phosphate had been reduced by the sodium borohydride. The relatively large loss of enzymic activity observed in this experiment suggested that the enzyme was being non-specifically inactivated by the large amount of sodium borohydride used; when only one-fifth as much sodium borohydride was used, more than 50 % of the activity was recovered (Table 4).

DISCUSSION

Physical properties of the enzyme. Ultracentrifugal studies (see the Appendix) of the enzyme show that it is monodisperse and has a molecular weight of about 760 000. Since this preparation studied contained 1 mole of pyridoxal 5'-phosphate/60 000 g. of protein, it must contain 13 moles of

pyridoxal 5'-phosphate/mole of enzyme. This preparation of enzyme catalysed the decarboxylation of 90 μ moles of L-aspartate/min./mg. of protein at pH 5.0 and 30°; hence its molecular activity was 69 300 moles of L-aspartate decarboxylated/min./mole of enzyme and its catalytic-centre activity was 5300 moles of L-aspartate decarboxylated/min./mole of pyridoxal 5'-phosphate.

The variable content of pyridoxal 5'-phosphate in different crystalline preparations of L-aspartate 4-carboxy-lyase is not readily explicable. Proteins, for example, bovine serum albumin (Dempsey & Christensen, 1962), are known to bind pyridoxal 5'-phosphate at sites that have no catalytic activity, and it is possible that an enzyme purified in the presence of added pyridoxal 5'-phosphate might bind some of the coenzyme at sites not concerned with the activity of the enzyme. However, since L-aspartate 4-carboxy-lyase has only one spectral peak, and since this peak is almost completely removed by the addition of a substrate analogue or high concentrations of the substrate, it seems likely that all of the bound pyridoxal 5'-phosphate is involved in the activity of the enzyme.

Mode of binding of pyridoxal 5'-phosphate. In all of the purified proteins containing pyridoxal 5'-phosphate that have been studied the carbonyl group of the pyridoxal 5'-phosphate is not free but is bound to the protein in one of two ways. In the acid pH range (pH 4.0–5.3) the spectra of such purified proteins show absorption maxima between 410 and 430 $m\mu$ (see Jenkins, 1961b; Snell, 1961). Reduction of this form of the enzyme with sodium borohydride results in a shift of the absorption maximum to 325 $m\mu$ (Hughes *et al.* 1962; Dempsey & Christensen, 1962). ϵ -Pyridoxyl-lysine has been isolated from hydrolysates of the sodium borohydride-reduction products of phosphorylase (Fischer, Kent, Snyder & Krebs, 1958), L-aspartate-2-oxoglutarate aminotransferase and L-homoserine

hydro-lyase (deaminating) (Fischer & Krebs, 1959), and the pyridoxal 5'-phosphate derivative of bovine serum albumin (Dempsey & Christensen, 1962). It is therefore probable that the pyridoxal 5'-phosphate forms a Schiff base linkage with the ϵ -amino group of lysine in the unreduced protein. From spectral studies of the pyridoxal-valine imine, Metzler (1957) concluded that the form absorbing at $414\text{ m}\mu$ had structure (I) (Fig. 8) in which the imino nitrogen of the Schiff base is hydrogen-bonded with the hydrogen of the phenolic group of pyridoxal. This conclusion is partly based on the observation, by Ferguson & Kelly (1951), that methylation of the phenolic group of salicylaldehydes, which are chemically similar to imines of pyridoxal 5'-phosphate, abolished the $420\text{ m}\mu$ absorption band of the unmethylated compounds. In certain proteins the Schiff base is converted at higher pH values into a more stable form that absorbs at $332\text{--}335\text{ m}\mu$ and that can no longer be reduced by sodium borohydride. Structures of type (III) (Fig. 8) have been proposed for these stable intermediates where $-X-$ may be $-\text{NH}-$, $-\text{S}-$ or $-\text{O}-$ (Kent, Krebs & Fischer, 1958; Dempsey & Christensen, 1962).

L-Aspartate 4-carboxy-lyase differs from all other purified proteins containing pyridoxal 5'-phosphate in having an absorption maximum at $360\text{ m}\mu$ instead of one near $420\text{ m}\mu$ in the acid pH range. Since the enzyme was reduced by sodium borohydride to give a protein-bound pyridoxyl derivative, it seems probable that the pyridoxal 5'-phosphate was bound to the unreduced protein in a Schiff base. This Schiff base might be of type (II) (Fig. 8), which differs from that suggested for other enzymes by not being hydrogen-bonded with the phenolic group of the pyridoxal 5'-phosphate due to some other, undetermined, feature of the protein.

L-Aspartate and its competitive inhibitor, DL-erythro- β -hydroxyaspartate, reacted with L-aspartate 4-carboxy-lyase to form an intermediate that absorbed at $325\text{ m}\mu$. After sodium borohydride treatment and dialysis of the erythro- β -hydroxyaspartate derivative of the enzyme,

enzymic activity was partially restored and most of the pyridoxal 5'-phosphate was in an unreduced state as evidenced by its reaction with phenylhydrazine. These results support a non-reducible structure, such as type (III) (Fig. 8), for the pyridoxal 5'-phosphate-erythro- β -hydroxyaspartate intermediate, where $-X-$ is the $-\text{NH}-$ of the amino group of erythro- β -hydroxyaspartate and the $-\text{NH}-$ is that of the ϵ -amino group of lysine.

Activation of the enzyme by keto compounds. Several modes of activation of L-aspartate 4-carboxy-lyase by keto acids have been proposed. Meister, Sober & Tice (1951) originally suggested that α -oxo acids activated enzyme-bound pyridoxamine 5'-phosphate by converting it into pyridoxal 5'-phosphate by transamination. Subsequent experiments (Nishimura *et al.* 1962) showed that all of the cofactor was present as pyridoxal 5'-phosphate. It was suggested that the α -oxo acids served to release the pyridoxal 5'-phosphate that might be bound in a form that was inaccessible to the substrate. The present observations, that both the substrate and substrate analogues formed derivatives of the protein-bound pyridoxal 5'-phosphate in the absence of pyruvate, are evidence against this hypothesis. The additional observation, that pyruvate caused the removal of the peak ascribed to an enzyme-substrate intermediate, suggests that pyruvate is involved in a second reaction. The effects of 'aging' of the enzyme and of high concentrations of aspartate on the pyruvate requirement remain unexplained. Although it is possible that 'aging' might alter the configuration of the protein and thus affect the reactivity of the enzyme-substrate intermediate, no change in the sedimentation coefficient of the 'aged' enzyme was observed (see the Appendix).

Suggested mechanism of aspartate decarboxylation. The observations reported in the present paper suggest that L-aspartate reacts initially with the protein-bound pyridoxal 5'-phosphate [structure (II) (Figs. 8 and 9)] to form a substituted aldimine derivative of type (III) (Fig. 9). It is possible that pyruvate facilitates L-aspartate decarboxylation by

Fig. 8. Structures that have been proposed for pyridoxal 5'-phosphate-protein derivatives. The hatched lines indicate the protein. $-X-$ may be $-\text{NH}-$, $-\text{S}-$ or $-\text{O}-$.

Fig. 9. Suggested mechanism of aspartate decarboxylation. The hatched lines indicate the protein.

displacing this substituted aldimine from the ϵ -amino group of lysine [structure (IV) (Fig. 9)]. It would be expected that subsequent tautomerization of the imine (IV) to a structure of type (V), in which the β -carboxyl group of aspartate would be in the β -position to a system of conjugated double bonds, would enable the loss of that carboxyl group as carbon dioxide to occur readily (cf. Braunstein, 1960). The resultant structure (VI) might undergo tautomerization and react with the ϵ -amino group of lysine in the enzyme to re-form structure (II): α -alanine would thus be released.

Since the evidence for the formation of a structure of type (III) from the enzyme and L-aspartate was obtained only under conditions in which the enzyme was inactive (i.e. in the absence of pyruvate and in the presence of high concentrations of L-aspartate), it might be argued that such a structure does not lie on the path of aspartate decarboxylation but was formed in a side reaction. However, the observations that the substrate analogue, *erythro*- β -hydroxyaspartate, is a competitive inhibitor (Fig. 3), and that it reacts with the enzyme to form a pyridoxal 5'-phosphate derivative with spectral characteristics indistinguishable from that observed with L-aspartate (Fig. 7b), favour the view that a structure of type (III) is a natural enzyme-substrate intermediate.

SUMMARY

1. An improved method of crystallizing L-aspartate 4-carboxy-lyase resulted in the formation of regular hexagonal plates.

2. The decarboxylation of L-aspartate (K_m 80 μ M) at pH 5.0 was competitively inhibited by DL-*erythro*- β -hydroxyaspartate (K_i 320 μ M) and DL-*threo*- β -hydroxyaspartate (K_i 11 μ M).

3. The enzyme was maximally active over a wide range of pH (3.5–6.0). Unlike other proteins containing pyridoxal 5'-phosphate, it exhibits a spectral peak at 360 m μ in this pH range.

4. Cyanide or hydroxylamine caused distinctive spectral changes when added to the enzyme, which was inhibited by these and other carbonyl reagents.

5. The activation of freshly prepared enzyme by 2-oxo acids and by glyoxal was not observed with 'aged' preparations unless high concentrations of L-aspartate were used.

6. Reduction of the enzyme with sodium borohydride inactivated it and produced a pyridoxyl derivative covalently bound to a group on the protein, tentatively identified as the ϵ -amino group of lysine.

7. L-Aspartate and *erythro*- β -hydroxyaspartate reacted with the enzyme to give derivatives with maximum extinction at 325 m μ . The *erythro*- β -hydroxyaspartate derivative was not reduced by sodium borohydride. Enzymic activity was partially recovered after dialysis.

8. Structures are suggested for the derivatives linking the enzyme or the substrate to pyridoxal 5'-phosphate; the role of such derivatives in the mechanism of L-aspartate decarboxylation is discussed.

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REFERENCES

- Braunstein, A. E. (1960). In *The Enzymes*, vol. 2, part A, p. 113. Ed. by Boyer, P. D., Lardy, H. & Myrbäck, K. New York and London: Academic Press Inc.
- Calvin, M. & Benson, A. A. (1949). *Science*, **104**, 140.
- Davison, A. N. (1956). *Biochim. biophys. Acta*, **19**, 131.
- Dempsey, W. B. & Christensen, H. N. (1962). *J. biol. Chem.* **237**, 1113.
- Ferguson, L. N. & Kelly, I. (1951). *J. Amer. chem. Soc.* **73**, 3707 (1951).
- Fischer, E. H., Kent, A. B., Snyder, E. R. & Krebs, E. G. (1958). *J. Amer. chem. Soc.* **80**, 99.
- Fischer, E. H. & Krebs, E. G. (1959). *Abstr. Pap. Amer. chem. Soc.*, 136th Meet., p. 24c.
- Hughes, R. C., Jenkins, W. T. & Fischer, E. H. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 1615.
- Jenkins, W. T. (1961a). *J. biol. Chem.* **236**, 1121.
- Jenkins, W. T. (1961b). *Fed. Proc.* **20**, 978.
- Jenkins, W. T., Orlowski, S. & Sizer, I. (1959). *J. biol. Chem.* **234**, 2657.
- Jenkins, W. T., Yphantis, D. A. & Sizer, I. (1959). *J. biol. Chem.* **234**, 51.
- Kent, A. B., Krebs, E. G. & Fischer, E. H. (1958). *J. biol. Chem.* **232**, 549.
- Kornguth, M. L. & Sallach, H. J. (1960). *Arch. Biochem. Biophys.* **91**, 39.
- Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Matsuo, Y. & Greenberg, D. M. (1958). *J. biol. Chem.* **230**, 561.
- Meister, A., Nishimura, J. S. & Novogradsky, A. (1962). *International Symposium on Biological and Chemical Aspects of Pyridoxal Catalysis, Rome* (in the Press).
- Meister, A., Sober, H. A. & Tice, S. V. (1951). *J. biol. Chem.* **189**, 577, 591.
- Metzler, D. E. (1957). *J. Amer. chem. Soc.* **79**, 485.
- Moore, S. & Stein, W. H. (1954). *J. biol. Chem.* **221**, 893.
- Nishimura, J. S., Manning, J. M. & Meister, A. (1962). *Biochemistry*, **1**, 442.
- Sakami, W. (1955). *Handbook of Isotope Tracer Methods*, p. 5. Cleveland: Department of Biochemistry, Western Reserve University School of Medicine.
- Sizer, I. W. & Jenkins, W. T. (1962). In *Methods in Enzymology*, vol. 5, p. 677. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Snell, E. E. (1961). *Ciba Found. Study Group no. 11: The Mechanism of Action of Water Soluble Vitamins*, p. 18. Ed. by de Reuck, A. V. S. & O'Connor, M. London: J. and A. Churchill.
- Wada, H. & Snell, E. E. (1962). *J. biol. Chem.* **239**, 2089.
- Wilson, E. M. (1963). *Biochim. biophys. Acta*, **67**, 345.
- Wilson, E. M. & Kornberg, H. L. (1963). *Biochem. J.* (in the Press).

APPENDIX

Ultracentrifuge Studies on Crystalline L-Aspartate 4-Carboxy-lyase

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The Spinco model E ultracentrifuge was used with the schlieren optical system. The enzyme was dissolved in 0.5 M-sodium acetate buffer, pH 7, and the protein concentration was determined refractometrically, by assuming a specific refractive increment of 1.85×10^{-3} .

Only one component was observed (see Fig. 2 of the main paper) which had a sedimentation coefficient $S_{20,w}$ of 19.8s at a concentration of 0.784 g./100 ml. of solution. The area under the curve of Fig. 2 (corrected for radial dilution) accounted for 90% of the protein present. The material was re-examined after keeping for 9 weeks ('aged' enzyme), but no change in sedimentation coefficient was observed.

The weight-average molecular weight was determined by the method of Archibald (1947) at a rotor speed of 2994 rev./min., a temperature of 19° and at a protein concentration of 0.784% (w/v). The partial specific volume was assumed to be 0.74 (the average of 31 values for proteins given by Svedberg & Pedersen (1940)), and the average molecular weight was found to be $760\,000 \pm 5\%$ (S.E.M. of five pictures).

REFERENCES

- Archibald, W. J. (1947). *J. phys. Colloid Chem.* **51**, 1204.
- Svedberg, T. & Pedersen, K. O. (1940). *The Ultracentrifuge*, p. 406. Oxford: The Clarendon Press.