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A Study of the Metabolism of L-αγ-Diaminobutyric Acid in a *Xanthomonas* Species

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1. L-αγ-Diaminobutyric acid is metabolized in *Xanthomonas* sp. to aspartic β-semialdehyde, aspartic acid and oxaloacetic acid. 2. Aspartic β-semialdehyde is formed from diaminobutyric acid by a pyruvate-dependent γ-transamination. 3. The transaminase has a pH optimum of 9 and exhibits a high degree of substrate specificity, as analogues of diaminobutyric acid and pyruvate are inert in the system. The transaminase is inhibited by carbonyl-binding agents such as hydroxylamine. 4. Aspartic acid is formed from aspartic β-semialdehyde by an NAD^+-dependent dehydrogenation. 5. The dehydrogenase has a pH optimum of 8·5 and is a thiol enzyme. It is specific for aspartic β-semialdehyde but analogues of NAD^+ such as 3-acetylpyridine–adenine dinucleotide and deamino-NAD are partly active in the system. 6. The significance of these reactions is discussed in relation to diaminobutyric acid metabolism in plants and mammalian systems.

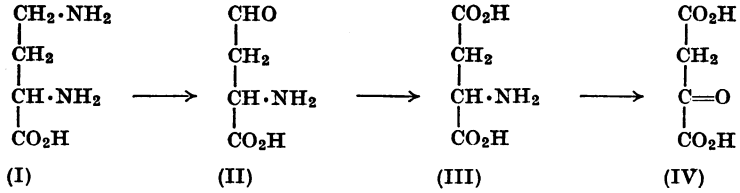
DABA* is a neurotoxic amino acid (O'Neal, Chen, Reynolds, Meghal & Koeppe, 1968) present in the free state in many plants (Ressler, Redstone & Erenberg, 1961; Bell, 1962; Van Etten & Miller, 1963; Bell & Tirimanna, 1965) and in the combined form in a variety of polypeptide antibiotics (Rebstock, 1960) and in bacterial cell walls (Perkins & Cummins, 1964). Studies *in vivo* with D- and L-αγ-diamino[2-^{14}C]butyric acid in the rat indicate a more rapid degradation of the L-isomer at both the α- and γ-carbon atoms, but the intermediates and enzymes involved have not been characterized (Mushawar & Koeppe, 1963). Nigam & Ressler (1966) have indicated that both homoserine and aspartic acid are good precursors of DABA in the plant *Lathyrus sylvestris* W. Seneviratne & Fowden (1968) have reported on the metabolism of DABA and αβ-diaminopropionic acid in a variety of plants.

The present paper deals with the metabolism of DABA in a bacterial system. We considered that a study of the metabolism of DABA in an aerobic organism capable of growing on DABA as the sole source of carbon and nitrogen would be helpful in elucidating the metabolic pathways concerned in its degradation as well as providing a good source of the enzymes catalysing the various reactions. Such a study has revealed that DABA (I) is metabolized by transamination to ABS (II), which is further converted into aspartic acid (III) and oxaloacetic acid (IV) (Scheme 1). A preliminary account of the work has been presented (Rajagopal Rao, Hariharan & Vijayalakshmi, 1968).

* Abbreviations: DABA, L-αγ-diaminobutyric acid; ABS, aspartic β-semialdehyde.

EXPERIMENTAL

Materials. DABA was synthesized on a large scale from L-glutamic acid by a modification (Gray & Paulus, 1964) of the original procedure of Adamson (1939) and purified by ion-exchange chromatography on a column of Amberlite CG-120(type I; H^+ form; 100–200 mesh). The precipitation of $BaSO_4$ was carried out at room temperature instead of in



Scheme 1.

boiling solutions as recommended by Gray & Paulus (1964) as, in our hands, their procedure resulted in racemization of DABA. DABA was converted into the hydrochloride and recrystallized from water–methanol. All samples of DABA were examined for homogeneity by paper chromatography and electrophoresis and only analytically pure samples were used for enzymic assays. DL-ABS was prepared by the method of Black & Wright (1955) by ozonization of DL-allylglycine (obtained from Cyclo Chemical Corp., Los Angeles, Calif., U.S.A.), and purified on a column of Dowex 50 (X8; H^+ form; 200–400 mesh). It invariably contained aspartic acid in small amounts. The concentration of L-ABS was determined by enzymic assay with ABS dehydrogenase in the presence of an excess of NAD^+. The concentration of DL-ABS was determined by chemical reduction with $NaBH_4$ to homoserine, which was determined in an amino acid analyser or by paper chromatography.

L-Glutamic acid was a product of E. Merck A.-G., Darmstadt, Germany. All other amino acids and oxo acids used for testing the substrate specificity of the enzymes were reagent-grade products of Sigma Chemical Co., St Louis, Mo., U.S.A., or of California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. γ-Acetyl-L-diaminobutyric acid was a gift from Dr N. L. Benoiton and was prepared as described by Benoiton & Leclerc (1965). γ-Amino-*n*-butyraldehyde was prepared by the method of Jakoby (1962) and kept frozen in an N_2 atmosphere. L-α-Aminoadipic semialdehyde was a gift from Dr V. Rodwell and was prepared enzymically (Basso, Rajagopal Rao & Rodwell, 1962). Acetaldehyde, *n*-propionaldehyde and *n*-butyraldehyde were commercial samples of the highest purity. Pyridoxal 5-phosphate was an E. Merck A.-G. product. NAD and NADP were products of Sigma Chemical Co. Other analogues of NAD were products of P-L Biochemicals Inc., Milwaukee, Wis., U.S.A. and were a gift from Dr V. Rodwell. *p*-Chloromercuribenzoic acid, *p*-chloromercuribenzenesulphonic acid and iodosobenzoic acid were products of Sigma Chemical Co. Analytical-grade reagents (British Drug Houses Ltd., Poole, Dorset, or E. Merck A.-G., or Sarabhai–Merck, Baroda, India) were used for the preparation of buffers for use in enzyme assays. DEAE-cellulose was a product of Sigma Chemical Co., and Sephadex of Pharmacia, Uppsala, Sweden.

Media for growth of organisms. The medium used for isolation of organisms by elective culture contained (per l.): 0·27g. of KH_2PO_4, 0·26g. of $MgSO_4,7H_2O$, 2·8mg. of $FeSO_4,7H_2O$, 1·5mg. of $MnSO_4$ and 2·1mg. of Na_2MoO_4. It was adjusted to pH 7 with NaOH and sterilized by autoclaving at 120° for 10min. The medium contained 0·2% neutralized DABA as the sole source of carbon and nitrogen. Solutions of DABA were sterilized by filtration in an all-glass bacteriological filter.

The basal medium for growth of the isolated *Xanthomonas* sp. was that described by Goodhue & Snell (1966). The isolated organism was maintained on this medium containing 2% agar. Liquid cultures were grown at 30° in the above medium in a New Brunswick Gyrorotary shaker, or, for large-scale growth, in a 20l. fermenter (with forced aeration), containing 15l. of the basal medium supplemented with 0·05% yeast extract. The yield of cells was approx. 1·5g. wet wt./l. of medium. *Bacillus polymyxa* A.T.C.C. 10401 was grown in the nutrient medium described by Gray & Paulus (1964). *Escherichia coli* strain K12 was grown in the medium described by Krishna & Krishnaswamy (1966).

Preparation of cell-free extracts. For most of the studies, extracts were prepared by grinding the cells with alumina (Alcoa-301, 1 part of cells to 2 parts of alumina) by the procedure of McIlwain (1948) and extracting with 2vol. of buffer (0·1M-tris–HCl or 0·1M-K_2HPO_4–NaH_2PO_4, pH 8·5) containing 1mM-EDTA. The extracts were centrifuged at 10000*g* for 30min. at 0–5° to remove unbroken cells and debris. Some preliminary experiments were carried out with extracts prepared by crushing cells in a Hughes (1951) press.

Manometric methods. Oxygen uptake by washed-cell suspensions was measured in conventional Warburg manometers at 37° with air as the gas phase.

Paper chromatography and electrophoresis. Chromatograms were run on Whatman no. 1 and no. 3MM paper with the following solvents: 1, butan-1-ol–acetic acid–water (2:1:1, by vol.); 2, phenol–water (4:1, v/v). Electrophoresis was carried out on Whatman no. 3MM paper at 1500v in a modified Michl (1951) apparatus with acetic acid–formic acid buffer, pH 1·8 (Rothman & Higa, 1962). Papers were dried, dipped in 0·2% ninhydrin in acetone and heated at 80° until the colours developed.

Ion-exchange chromatography. The following ion-exchange resins were used: Dowex 50W (X4; H^+ form; 100–200 mesh), Dowex 50W (X8; H^+ form; 200–400 mesh), Dowex 1 (X8; acetate form; 100–200 mesh) and Amberlite CG-120 (type 1; H^+ form; 100–200 mesh). Before use the cation-exchange resins were repeatedly cycled through Na^+ and H^+ forms and the anion-exchange resins through Cl^- and acetate forms and then exhaustively washed with water. Identification and determination of some of the amino acids were carried out in an amino acid analyser with Beckman-grade spherical resin PA28 and the buffers recommended by the manufacturers.

Determination of protein. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Ovalbumin was used as standard. To follow the purification of the enzymes on DEAE-cellulose or Sephadex columns, protein was also determined by the method of Warburg & Christian (1941).

Enzyme assays

Transaminase reaction (*DABA + pyruvate* $\rightleftharpoons$ *ABS + alanine*). (*a*) Chromatographic procedure. Reaction mixtures contained in 1ml.; 20μmoles of amino acid (DABA or other amino acid), 20 μmoles of oxo acid (pyruvate or other oxo acid), 20 μmoles of buffer (K_2HPO_4–NaH_2PO_4 or tris–HCl), pH 8·5, and 0·1–0·2ml. of enzyme (0·3–1mg. of protein). After incubation for 30min. at 37°, the reaction mixture was deproteinized by adding 0·1ml. of 16% (w/v) $HClO_4$ and centrifuging. Blanks contained boiled enzyme together with all the other components. For the determination of alanine, 10–20μl. samples of supernatant were chromatographed along with standards of alanine on Whatman no. 1 paper with solvent 1. After development of colour with ninhydrin, the alanine spots were eluted with ethanolic $CuSO_4$ and measured colorimetrically at 540nm. (Giri, Radhakrishnan & Vaidyanathan, 1952). In this assay, specific activity is defined as nmoles of alanine formed/hr./mg. of protein. The transaminase reaction was also studied in the reverse direction by incubating ABS and L-alanine in the presence of enzyme. The DABA formed was identified by paper electrophoresis at pH 1·8. As there was a progressive non-enzymic decom-

position of ABS at alkaline pH, the results were not quantitative.

(*b*) Spectrophotometric procedure. The transaminase reaction was also studied by an optical assay system that utilized two reactions:

$$\text{DABA} + \text{pyruvate} \rightarrow \text{ABS} + \text{alanine} \quad (1)$$

$$\text{ABS} + \text{NAD}^+ + \text{H}_2\text{O} \rightarrow \text{aspartic acid} + \text{NADH} \quad (2)$$

Sum: $\text{DABA} + \text{pyruvate} + \text{NAD}^+ + \text{H}_2\text{O} \rightarrow \text{aspartic acid} + \text{alanine} + \text{NADH}$ (3)

Reaction (1) is catalysed by transaminase and reaction (2) by ABS dehydrogenase.

The reaction mixture contained (in 3ml.); 100 μmoles of buffer (tris–HCl or K_2HPO_4–NaH_2PO_4), pH 8·5, 20 μmoles of pyruvate, 0·6 μmole of NAD^+ and an excess of purified ABS dehydrogenase. After addition of a source of transaminase, the reaction was started by the addition of 20 μmoles of DABA and the increase in E_{340} was measured every 30sec. for 5min. All reactions were run at room temperature (25–28°) with two different concentrations of transaminase.

One unit of enzyme activity is defined as 1 nmole of NADH formed/min. under the above assay conditions. Specific activity is defined as the number of units/mg. of protein. The change in extinction from ½ to 1½ min. is used to calculate the specific activity. During this interval, the rate is linear and proportional to enzyme concentration.

ABS dehydrogenase. The assay system contained (in 3ml.); 100 μmoles of buffer (tris–HCl or K_2HPO_4–NaH_2PO_4), pH 8·5, 2 μmoles of NAD^+, 2 μmoles of DL-ABS and 100–300 μg. of enzyme protein. The increase in E_{340} was measured every 30sec. for 5min. The units of activity and specific activity are the same as those described in the spectrophotometric assay of transaminase.

Other enzymes. D-Amino acid oxidase (EC 1.4.3.3) was extracted from an acetone-dried powder of sheep kidney and assayed by the method of Burton (1955). Malate dehydrogenase (EC 1.1.1.37) and 'malic' enzyme (EC 1.1.1.40) were assayed as described by Ochoa (1955). Homoserine dehydrogenase (EC 1.1.1.3) was assayed by the procedure of Black & Wright (1955). Aspartate-2-oxoglutarate aminotransferase (EC 2.6.1.1) was determined by the chromatographic assay procedure described above for DABA transaminase, except that aspartate and α-oxoglutarate were used as substrates.

Identification of L-alanine as product of transamination reaction. In a round-bottomed flask, 2m-moles of DABA and 2m-moles of pyruvate were dissolved in 30ml. of water (pH 8·0) and $NaHSO_3$ was added to give a final concentration of 0·01M. Crude enzyme (step 1) (50ml. from 25g. of bacterial cells) was added. The flask was evacuated and the reaction mixture incubated at 24–25° for 24hr. The reaction was terminated by addition of 1ml. of 70% (w/v) $HClO_4$, the protein removed by centrifuging and the solution neutralized with KOH. Paper electrophoresis of a small sample showed the presence of alanine. Reactions carried out in the absence of DABA revealed no formation of alanine. The bulk of the material was applied to a Dowex 50 (H^+ form 100–200 mesh) column; (15cm. × 2cm.) and washed with 250ml. of water. The column was eluted with 0·5M-HCl and 10ml. fractions were collected. Alanine was found in fractions 10–17, which were combined and concentrated to dryness *in vacuo*. The residue was dissolved in water and applied to a Dowex 1 (acetate form; 100–200 mesh) column, which was washed with 100ml. of water. The water washings were concentrated to dryness *in vacuo* and the residue was dissolved in water. The concentration of alanine was determined chromatographically. The yield was 80mg. (0·9m-mole).

The isolated alanine was inert to D-amino acid oxidase in manometric experiments and had the same chromatographic mobility as authentic alanine in solvent systems 1 and 2.

RESULTS

Isolation and identification of bacterial species. The organism was isolated from soil by elective growth on the medium described, which contained DABA as sole source of carbon and nitrogen, followed by plating out on agar medium of the same composition.

The organism isolated showed the following characteristics, similar to those of *Xanthomonas* sp. (Breed, Murray & Smith, 1957); it was a Gram-negative cocco-bacillus, sluggish to actively motile with one to three polar flagella at one end; agar colonies were small, pale yellow, transparent with entire margin, with circular form, concave elevation and smooth surface; it turned nutrient broth turbid and produced a yellow insoluble pigment; it turned milk alkaline; it produced acid but no gas from glucose and sucrose and neither acid nor gas from lactose; neither indole nor hydrogen sulphide was produced; Methyl Red and Voges–Proskauer tests were negative; cellulose and gelatin were not decomposed; catalase test was positive and nitrates were reduced to nitrites.

The organism was not identified to the species level, as plant-pathogenicity tests were necessary for this.

Manometric studies. Cell suspensions of *Xanthomonas* sp. actively oxidized DABA and a number of related compounds (Fig. 1*a*). No detectable metabolites, other than alanine, accumulated in the reaction mixture when DABA was incubated with cell suspensions. When cells were grown on lysine or glutamate, DABA was poorly oxidized (Figs. 1*b* and 1*c*).

Experiments with cell-free extracts. To test for the formation of alanine, cell-free extracts were incubated with DABA and pyruvate, and the products of the reaction were examined by paper chromatography (with solvents 1 and 2) and high-voltage electrophoresis at pH 1·8. Alanine was formed and its concentration increased with time (Fig. 2). The formation of alanine was dependent on the presence of pyruvate and DABA. Reaction mixtures containing DABA, pyruvate and enzyme progressively developed a colour ($\lambda_{max.}$ 510nm.) (Fig. 2). When the same reaction was carried out under anaerobic conditions in a Thunberg tube or in the presence of 0·02M-sodium bisulphite, no colour was formed. It was thus established that colour formation was due

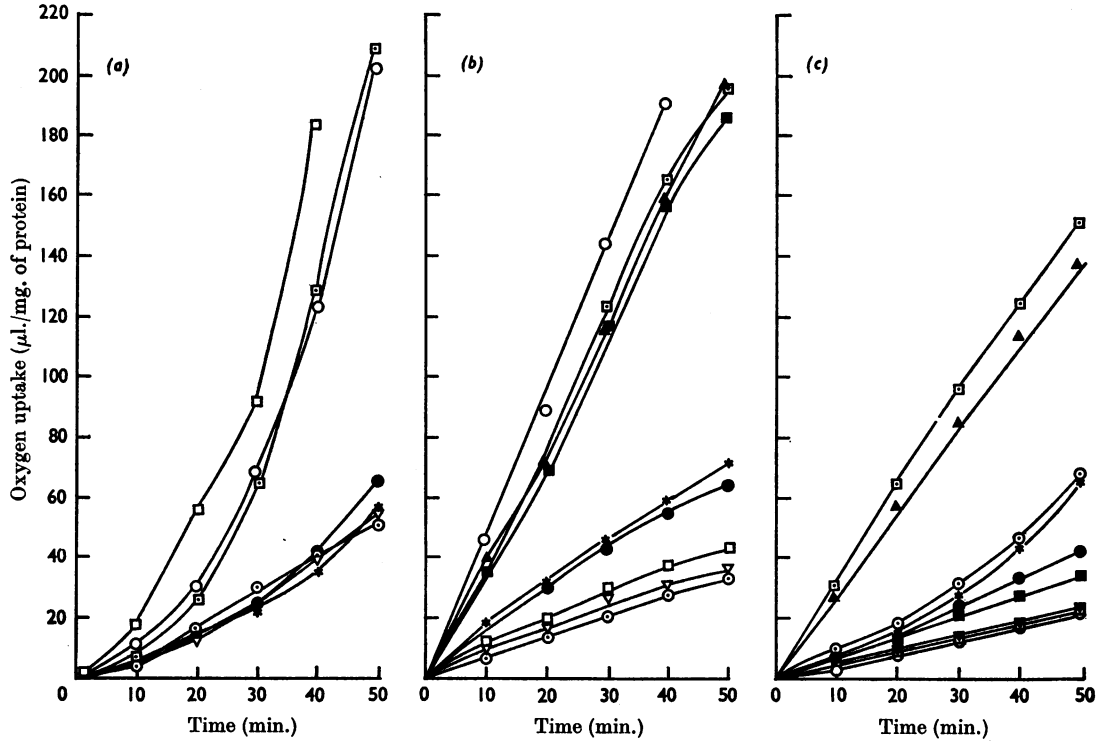


Fig. 1. Oxidation of various compounds by *Xanthomonas* sp. grown on (*a*) DABA, (*b*) lysine and (*c*) glutamate. The main compartment of the Warburg flask contained (in 3ml.) 100μmoles of K_2HPO_4–NaH_2PO_4 buffer, pH 7·2, and 0·5ml. of cell suspension (5mg. of protein). The centre well contained 0·2ml. of 10% KOH to absorb CO_2. After equilibration (10min.) at 37°, the substrate (20μmoles) was tipped from the side arm. Substrates: ⊙, endogenous; □, DABA; ▽, DL-$\alpha\beta$-diaminopropionate; ▼, L-glutamate; ■, L-lysine; ○, L-γ-aminobutyrate; ●, L-homoserine; ⁎, propionate; ⊡, L-aspartate; ▲, β-alanine.

to the aerobic lability of ABS, which was one of the products formed by transamination. Synthetic ABS also forms a coloured compound ($\lambda_{max.}$ 510nm.) under aerobic conditions at alkaline pH. The colour formation is a non-enzymic reaction.

Purification of transaminase and ABS dehydrogenase. The purification of the enzymes involved in the metabolism of DABA was followed by the spectrophotometric assay.

Step 1: Preparation of crude extract. *Xanthomonas* cells (25g. wet wt.) were ground with 50g. of alumina (Alcoa-301) in two portions in a chilled mortar. The grinding was continued until the mass was viscous and it was then extracted with 2vol. of 0·1M buffer (tris–HCl or K_2HPO_4–NaH_2PO_4), pH 8·5, containing 1mM-EDTA and 1mM-GSH. The extract was centrifuged at 10000*g* for 30min. and the clear supernatant (50ml.) used for further studies ('crude extract').

Step 2: Streptomycin sulphate treatment. Streptomycin sulphate (500mg.) was dissolved in 5ml. of water and slowly added to the chilled (0–5°) crude extract. The precipitate was removed by centrifuging.

Step 3: Ammonium sulphate fractionation. To the supernatant (50ml.) from the previous step, ammonium sulphate (12·1g.) was gradually added to give 40% saturation and the precipitate was discarded. To the supernatant (50ml.) from the 40%-saturation step, ammonium sulphate (6·6g.) was added to give 60% saturation. The precipitate was collected and dissolved in 10ml. of 0·1M buffer ('40–60%-saturation ammonium sulphate fraction'). To the supernatant (50ml.) from the 60%-saturation step, ammonium sulphate (7g.) was added to give 80% saturation. The precipitate was collected and dissolved in 10ml. of 0·1M buffer ('60–80%-saturation ammonium sulphate fraction').

Step 4: Removal of low-molecular-weight materials. The enzyme solution ('40–60%-saturation ammonium sulphate fraction') was either

dialysed against 10vol. of 0·01M buffer (tris–HCl or K_2HPO_4–NaH_2PO_4), pH 8·5, in the cold or passed through a Sephadex G-50 column.

The results of a typical fractionation are given in Table 1. Most of the experiments on the transaminase and dehydrogenase were carried out with the partially purified enzyme. Attempts to purify the enzymes (40–60%-saturation ammonium sulphate fraction) further on DEAE-cellulose or by adsorption on calcium phosphate gel led to low recovery of the activities and no apparent increase in the specific activities.

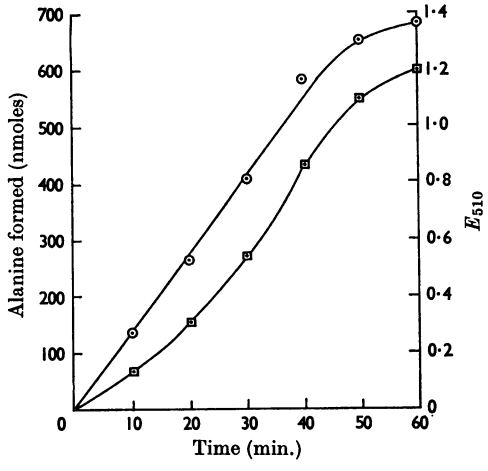


Fig. 2. ⊙, Time-course of transamination reaction. Tubes containing the standard reaction mixture (for chromatographic assay) were incubated at 37° for various times and the alanine formed was determined by paper chromatography. ⊡, Time-course of development of colour at 510nm. Tubes containing standard reaction mixture (for chromatographic assay) were incubated at 37° for various times and deproteinized by addition of 2ml. of ethanol. After centrifugation, the E_{510} of the supernatant was determined.

Diaminobutyrate–pyruvate transaminase

Nature of the group participating in transamination. DABA can lose either the α-amino group or the γ-amino group during transamination (Scheme 2), forming either ABS or γ-amino-α-oxobutyric acid. These two compounds can be distinguished by the nature of products they form upon oxidation (with hydrogen peroxide) and reduction (with sodium borohydride). When such experiments were carried out with appropriate controls, it was found that homoserine was invariably formed on reduction and aspartic acid on oxidation. These compounds were identified by paper and ion-exchange chromatography. Neither γ-amino-α-hydroxybutyric acid nor β-alanine was detected in these experiments. Further proof that the γ-amino group was lost during transamination was obtained by trapping the ABS as the bisulphite addition product. The addition product was applied to a column of Dowex 50 (X4; H^+ form; 100–200 mesh) and the water washings were freeze-dried. The solid material was dissolved in water and a sample was allowed to react with acid ninhydrin by using the method of Work (1957). The absorption spectrum

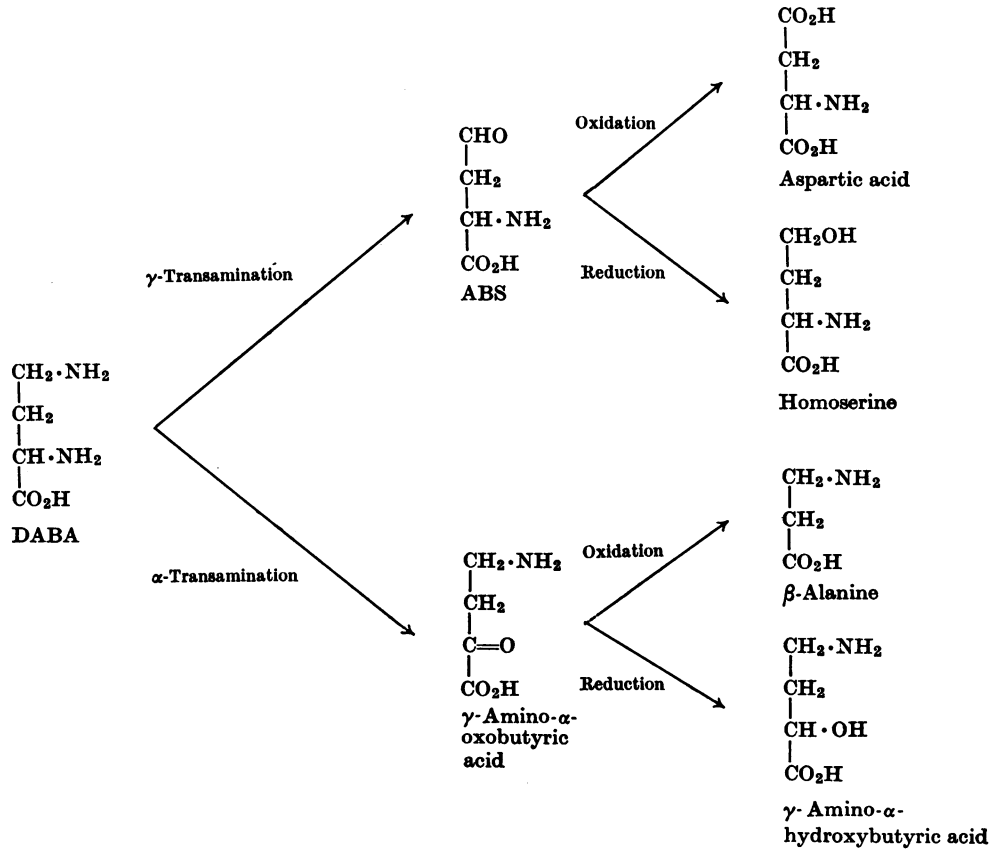
Table 1. *Purification of DABA transaminase and ABS dehydrogenase*

A 12·5g. (wet wt.) portion of *Xanthomonas* cells was used as the starting material. Enzyme activities are expressed in units of nmoles/min.

			DABA transaminase			ABS dehydrogenase		
Step no.	Method	Total protein (mg.)	Sp. activity (units/mg. of protein)	Total activity (units)	Recovery (%)	Sp. activity (units/mg. of protein)	Total activity (units)	Recovery (%)
1	Crude extract	175·5	208·1	36520	100	423·6	74340	100
2	Streptomycin sulphate treatment	148·5	228·3	33910	93	614·9	91330	123*
3	(*a*) 0–40%-satn. $(NH_4)_2SO_4$ fraction	13·6	125·0	1700	5	773·0	10510	14
	(*b*) 40–60%-satn. $(NH_4)_2SO_4$ fraction	46·2	492·0	22720	62†	1036·0	47880	64†
	(c) 60–80%-satn. $(NH_4)_2SO_4$ fraction	19·95	55·2	1102	3	156·4	3120	4

* The cause of the apparent increase in the ABS dehydrogenase activity after streptomycin sulphate treatment is not known.

† This fraction was dialysed against 50vol. of 0·01M-K_2HPO_4–NaH_2PO_4 buffer, pH 8·5, and used for most of the studies.



Scheme 2.

of the resulting product was identical with that obtained with synthetic ABS ($\lambda_{max.}$ 390nm.).

Additional evidence for a γ-transamination was provided by scaling-up the spectrophotometric assay procedure for transaminase to a preparative scale and identifying the aspartic acid formed by ion-exchange chromatography (in an amino acid analyser) and by paper chromatography. Further, γ-acetyl-L-diaminobutyric acid was inert in the transamination reaction, indicating that a free amino group in the γ-position is necessary.

Optimum pH *and substrate specificity.* The transaminase has optimum pH 9 (by the chromatographic assay procedure). The reaction exhibits a high degree of substrate specificity, as L-lysine, L-ornithine, DL-αβ-diaminopropionic acid and β-alanine could not replace DABA in the assay system. α-Oxoglutarate, oxaloacetate, glyoxalate, phenylpyruvate and *p*-hydroxyphenylpyruvate could not replace pyruvate in the assay system.

Effect of inhibitors. The presence of EDTA in the transaminase assay (by the chromatographic procedure) had no appreciable effect on the formation of alanine, indicating that heavy-metal ions were not needed for the reaction. The enzyme was inhibited by carbonyl-binding agents, particularly hydroxylamine, which inhibited the enzyme by about 87% at a concentration of 3·3mM (Table 2). When the enzyme was dialysed against a solution of sodium borohydride, it lost all of its activity. Activity of the hydroxylamine-inhibited enzyme or the borohydride-treated enzyme could not be restored by addition of pyridoxal phosphate. When pyridoxal phosphate was added to the dialysed enzyme, there was no increase in activity.

Aspartic β-semialdehyde dehydrogenase

Cell-free extracts have an active ABS dehydrogenase that converted ABS into aspartic acid in the presence of NAD^+. The enzyme has been partially purified (Table 1).

Optimum pH *and substrate specificity.* The ABS dehydrogenase has optimum pH 8·5. The enzyme is

Table 2. *Effect of inhibitors on DABA transaminase reaction*

The standard assay system (chromatographic procedure) was used. The enzyme was preincubated with the inhibitor in the presence of buffer for 10 min. and other components of the assay system were then added. For the effect of $NaBH_4$ on the enzyme, the enzyme (10 mg.) was dialysed for 30 min. against 5 mM-$NaBH_4$ and the pH was maintained near 8 by addition of dil. acetic acid. The enzyme was redialysed for 4 hr. against several changes of 0·1 M-tris–HCl buffer, pH 8·5, and then assayed for activity both in the absence and presence of pyridoxal phosphate.

Inhibitor	Concn. (mM)	Alanine formed (nmoles/hr.)	Inhibition (%)
None	—	60·5	0
EDTA	10·0	60·5	0
Hydroxylamine hydrochloride	3·3	7·85	87·0
	0·33	25·0	58·3
	0·033	50·5	16·6
	0·0033	60·5	0
NaCN	3·3	60·5	0
Semicarbazide hydrochloride	20·0	10·3	83·0
	10·0	21·8	63·0
$NaBH_4$	5·0	0	100
$NaBH_4$-treated enzyme + 10 μg. of pyridoxal phosphate	5·0	0	100

Table 3. *Effect of inhibitors on ABS dehydrogenase*

The standard assay system for ABS dehydrogenase was used. The enzyme was preincubated with the inhibitor in the presence of buffer, pH 8·5, for 5 min. and other components of the assay system were then added.

Inhibitor	Concn. (mM)	NADH formed (nmoles/min.)	Inhibition (%)
None	—	309	0
+ GSH		309	0
p-Chloromercuribenzoate	0·33	0	100
	0·033	48·5	84
	0·0033	260·0	12·5
p-Chloromercuribenzenesulphonate	0·33	0	100
	0·033	9·6	97
	0·0033	101	66
Iodosobenzoate	0·33	33·8	89
	0·033	222	28
	0·0033	285	7·5

specific for ABS, as aliphatic aldehydes such as acetaldehyde, *n*-propionaldehyde, *n*-butyraldehyde and amino aldehydes like γ-amino-*n*-butyraldehyde and L-α-aminoadipic semialdehyde are inactive. Among a number of nucleotide analogues only 3-acetylpyridine–adenine dinucleotide and deamino-NAD^+ are active. At a concentration of 0·23 mM, they are about 35–45% as active as NAD^+. Only the L-isomer of ABS was converted into aspartic acid when DL-ABS was used as the substrate, as indicated by the amount of NADH formed. It has been shown above (see under 'Nature of group participating in transamination') that aspartic acid was the product of dehydrogenation of ABS. No homoserine dehydrogenase activity could be demonstrated in extracts of *Xanthomonas* sp. grown on DABA.

Effect of inhibitors. ABS dehydrogenase is strongly inhibited by thiol-binding agents such as *p*-chloromercuribenzoate and *p*-chloromercuribenzenesulphonate (Table 3). These compounds at concentrations of the order of 0·1 mM completely inhibit the reaction.

Substrate affinities. The K_m values were calculated from Lineweaver–Burk plots, and are $2{\cdot}1 \times 10^{-4}$ M for NAD^+ and 1×10^{-3} M for ABS.

Reversibility of reaction. Attempts to reverse the dehydrogenase reaction by using aspartic acid and NADH were unsuccessful. It is well known that the conversion of an acyl group into an aldehyde group in biological systems involves prior activation of the group by either ATP alone or ATP and CoA, followed by reduction.

Further metabolism of aspartic acid. The aspartic

acid formed from ABS is converted into oxaloacetic acid by the aspartate–2-oxoglutarate aminotransferase present in the cell-free extracts. Oxaloacetic acid is further metabolized by malate dehydrogenase or 'malic' enzyme. The activities of these enzymes are: aspartate–2-oxoglutarate aminotransferase, 2·5μmoles of glutamate formed/hr./mg. of protein; malate dehydrogenase, 174 nmoles of NADH formed/min./mg. of protein; 'malic' enzyme, 60nmoles of NADPH formed/min./mg. of protein.

DISCUSSION

The present work has indicated that there are enzymes capable of metabolizing DABA in a *Xanthomonas* sp. grown on DABA. The first step in the metabolism of DABA is a γ-transamination to form ABS. The DABA transaminase reaction is reversible, but the lability of ABS at alkaline pH values precluded decisive experiments with unlabelled material. The transaminase reaction has obvious similarities to the δ-transamination of ornithine, which yields glutamic γ-semialdehyde (Meister, 1965). It is possible that DABA is formed in plants and a few bacteria by reversal of the transaminase reaction. The results of Nigam & Ressler (1966) during isotopic studies with *Lathyrus sylvestris* are consistent with such a hypothesis. The reactions leading to the formation of ABS from aspartic acid and homoserine have been described in yeast by Black & Wright (1955), in plants by Naylor, Rabson & Tolbert (1958) and Sasoka (1961) and also in *Bacillus polymyxa* by Gray & Paulus (1964). Our attempts to demonstrate the DABA transaminase reaction in *B. polymyxa* or *E. coli* K12 were unsuccessful, but this may be due to differences in strain of the organisms used or growth conditions.

The strong inhibition of DABA transaminase by carbonyl-binding agents such as hydroxylamine indicates that carbonyl groups are involved in the reaction. It is known that pyridoxal phosphate is the coenzyme in the mammalian ornithine transaminase (Tsai & Jenkins, 1968). The DABA transaminase is not sufficiently pure for us to say whether pyridoxal phosphate is involved as the coenzyme. The mammalian metabolism of DABA involves both the α- and γ-amino groups, as both aspartic acid and β-alanine appear in the urine (Mushawar & Koppe, 1963). Our attempts to detect DABA transaminase and ABS dehydrogenase in extracts of rat liver and kidney were unsuccessful. Attempts to induce the enzymes in rats by intraperitoneal injection of DABA were negative. These failures may be due to the fact that the activities of the enzymes are too low to be detected by non-isotopic assay procedures, or perhaps the metabolism of DABA in mammalian tissues involves oxidative deamination or other similar reactions.

The second step in the metabolism of DABA in *Xanthomonas* sp. is an NAD^+-dependent dehydrogenation of ABS to form aspartic acid. ABS dehydrogenase exhibits a high degree of substrate specificity. This is in contrast with glutamic γ-semialdehyde dehydrogenase (Strecker, 1960) and γ-hydroxyglutamic semialdehyde dehydrogenase (Adams & Goldstone, 1960), both of which have a broad substrate specificity. No homoserine dehydrogenase could be demonstrated in the *Xanthomonas* extracts, suggesting that ABS is not metabolized by this pathway.

Further metabolism of aspartic acid formed from ABS involves the well-known aspartate–2-oxoglutarate aminotransferase reaction, and this activity could be readily demonstrated in *Xanthomonas* extracts. The extracts also have an NAD^+-dependent malate dehydrogenase, but the activity of $NADP^+$-dependent 'malic' enzyme is low. The presence of all these enzymes enables the bacteria to metabolize DABA rapidly.

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