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FORMATION OF β-CYANOALANINE AND PYRUVATE BY *ACACIA GEORGINAE*

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Abstract—Pyruvate is formed on incubation of L-cysteine with acetone powder preparations of *Acacia georginae* but in the presence of cyanide, β-cyanoalanine is produced and pyruvate production is highly depressed The pH optimum for pyruvate production is 8 5 In the presence of fluoride (1 5 mM), the pH profile is unchanged and in the presence of cyanide (1 5 mM), minimal pyruvate production occurs at pH 8 5 Although addition of pyridoxal phosphate had no influence on pyruvate or β-cyanoalanine production, these processes were prevented by sodium borohydride, an inhibitor of pyridoxal enzymes Neither L-serine nor *O*-acetyl-L-serine serve as alternative substrates for pyruvate production. β-Fluoroalanine was not detected on incubating fluoride with an enzyme preparation from *A georginae* acetone powders

INTRODUCTION

IN RECENT years, numerous reports have described the occurrence of novel amino acids of the β-substituted alanine type.[1,2] The biosynthesis of many of these has been shown to involve a pyridoxal phosphate-dependent α-β elimination of hydrogen sulphide, water, or acetic acid from the substrates cysteine, serine, or *O*-acetyl serine respectively and the incorporation of a different nucleophile by addition or exchange reactions [3–6] The present communication reports on the biosynthesis of β-cyanoalanine in *Acacia georginae*, F M Bailey from cysteine and cyanide by such processes

It has been proposed[7] that the formation of fluoroacetic acid in *A georginae* initially involves a similar α-β elimination and incorporation of the nucleophilic fluoride ion to produce β-fluoroalanine The ability of the plant preparations to incorporate the fluoride ion by such a process was also investigated In the presence or absence of fluoride, cysteine was converted to pyruvate Replacement of fluoride by cyanide almost abolished pyruvate formation and led to the production of β-cyanoalanine

RESULTS

The α-β eliminase in *A georginae* catalyzes the conversion of cysteine to pyruvate with a mean stoichiometry of 96% for cysteine added. Seven determinations up to 1 μmol were made.

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[2] BELL, E A (1971) in *Chemotaxonomy of the Leguminosae* (HARBORNE, J B , BOULTER, D and TURNER, B L , eds), p 179, Academic Press, London
[3] BLUMENTHAL, S G , HENDRICKSON, H R , ABROL, Y. P and CONN, E E (1968) *J Biol Chem* **243**, 5302
[4] MURAKOSHI, I , KURAMOTO, H and HAGINAWA, J (1972) *Phytochemistry* **11**, 177
[5] HENDRICKSON, H R and CONN, E E (1969) *J Biol Chem* **244**, 2632
[6] DUNNILL, P M and FOWDEN, L (1963) *J Exp Botany* **14**, 237.
[7] MEAD, R J and SEGAL, W (1972) *Australian J Biol Sci* **25**, 327

Pyridoxal phosphate (PALP), at concentrations ranging from 0 to 2 mM, failed to enhance the enzyme activity The addition of a trace of sodium borohydride, which inhibits pyridoxal enzymes, abolished pyruvate production No pyruvate was produced when L-cysteine was replaced by either *O*-acetyl-L-serine (OAS) or L-serine in acetone powder incubations in the pH range 7 4–9 7

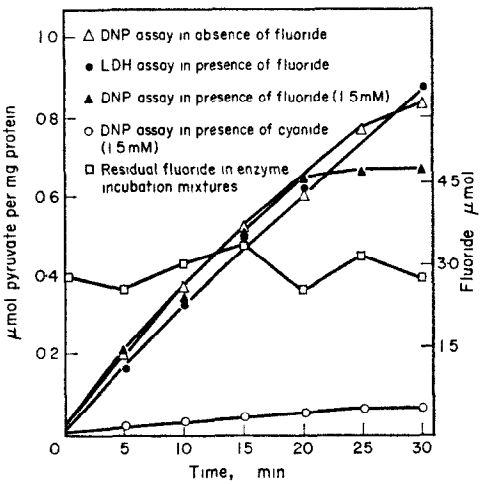

FIG 1 PRODUCTION OF PYRUVATE FROM CYSTEINE IN THE PRESENCE AND ABSENCE OF FLUORIDE AND CYANIDE AT pH 8 5

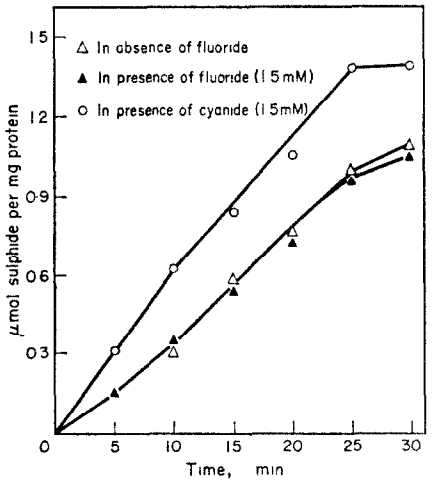

FIG 2 HYDROGEN SULPHIDE ELIMINATION IN PRESENCE AND ABSENCE OF FLUORIDE AND CYANIDE AT pH 8 5

As the 2,4-dinitrophenylhydrazone (DNP) assay is not specific for pyruvate, the incubation mixtures were extracted with toluene after DNP formation (see Experimental) This results in the selective extraction of the less polar DNP derivatives, including pyruvate Subsequent chromatography allows isolation of the *syn*-and- *anti*-forms of pyruvate DNP Pyruvate production was also confirmed using lactic dehydrogenase (LDH) as an assay

The relationship between pH and pyruvate production in the presence or absence of fluoride was almost identical, with an optimum at pH 8 5 There was a marked depression in pyruvate production in the presence of cyanide to 10% of the activity at pH 8 5 in its absence No clear pH optimum was observed The time course of pyruvate production from cysteine was also investigated in the presence and absence of fluoride and cyanide, and the results are presented in Fig 1

In the presence of authentic pyruvate, cyanide had no influence on the formation of pyruvate DNP and the depression in pyruvate production in the presence of cyanide was confirmed using LDH

In order to further investigate the eliminase reaction attempts were made to measure hydrogen sulphide by a technique in which methylene blue is synthesized in its presence,[3] but these estimations did not correlate with those obtained on calibration of the method with standard amounts of sodium sulphide For this reason, the H_2S formed in the stoichiometric conversion of cysteine to pyruvate was used in establishing a standard curve Examination of hydrogen sulphide elimination in the presence and absence of cyanide and fluoride indicated that the α-β elimination reaction was initially almost twice as fast in the presence of cyanide (Fig 2)

The β-cyanoalanine synthase activity of blue lupin, sorghum and common vetch, has been measured by assay of hydrogen sulphide elimination using cysteine as substrate [3] In the present study with *A georginae*, cyanide was shown to be converted to β-cyanoalanine by use of an automated amino acid analyzer with reference to authentic β-cyanoalanine β-Cyanoalanine produces a characteristic green colouration with ninhydrin The same colour reaction was observed with the amino acid product derived enzymatically In incubations conducted both in the presence and absence of cyanide the initial cysteine concentrations were reduced by approximately 90% when compared to an enzyme-inactivated blank

Although it has been reported that numerous species convert β-cyanoalanine to asparagine,[3,8] there was no evidence that this process occurred in *A georginae* incubations as assessed by amino acid analysis

There was no evidence for the production of β-fluoroalanine in incubations containing fluoride ion under conditions favouring the formation of β-cyanoalanine, and the fluoride ion concentrations remained essentially unchanged over time-course experiments (Fig 1) and pH studies and in experiments where OAS and serine replaced cysteine as substrate Fluoropyruvate DNP is toluene-soluble and is also similar in its chromatographic properties to pyruvate DNP Moreover fluoropyruvate is an acceptable substrate to LDH [9] To further confirm that fluoride had not been incorporated, the DNP isolated from an incubation carried out in the presence of fluoride was examined by MS The mass spectrum was devoid of a hydrogen fluoride signal at $m/e = 20$, which proved to be a strong signal in the mass spectrum of authentic fluoropyruvate DNP

DISCUSSION

The production of pyruvate from amino acid substrates in plant preparations has previously been reported [10,11] A recent communication,[10] describes the production of pyruvate from *O*-acetyl-L-serine by *Brassica* species, by an α-β elimination process Cyanide was found to produce an 85% inhibition of pyruvate production but the nature of the inhibition and the possibility of β-cyanoalanine formation was not discussed

In the present study the conversion of cysteine (I) to pyruvate (II) in the absence of cyanide and the formation of β-cyanoalanine (III) instead of pyruvate in the presence of cyanide, suggests that the α-β elimination reaction and nucleophile substitution may be associated through the common intermediates (IV) and (V)

The alternative pathways described above are analogous to those described in *Leucaena* seedlings where mimosine (β- *N*-(3-hydroxy-4-pyridonyl) alanine) is degraded to pyruvate, with the elimination of 3,4-dihydroxypyridine [11] However, in the presence of the nucleophile methylmercaptan, *S*-methylcysteine is formed instead of pyruvate [12]

Several β-substituted alanines have been isolated from *A georginae* [13] These include β-acetylaminoalanine, albizine (β-ureidoalanine), and djenkolic acid (3,3'-(methylenedithio) dialanine) The biosynthesis of these substances in *A georginae* has not been reported

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[10] MAZELIS, M and FOWDEN, L (1972) *Phytochemistry* **11**, 619
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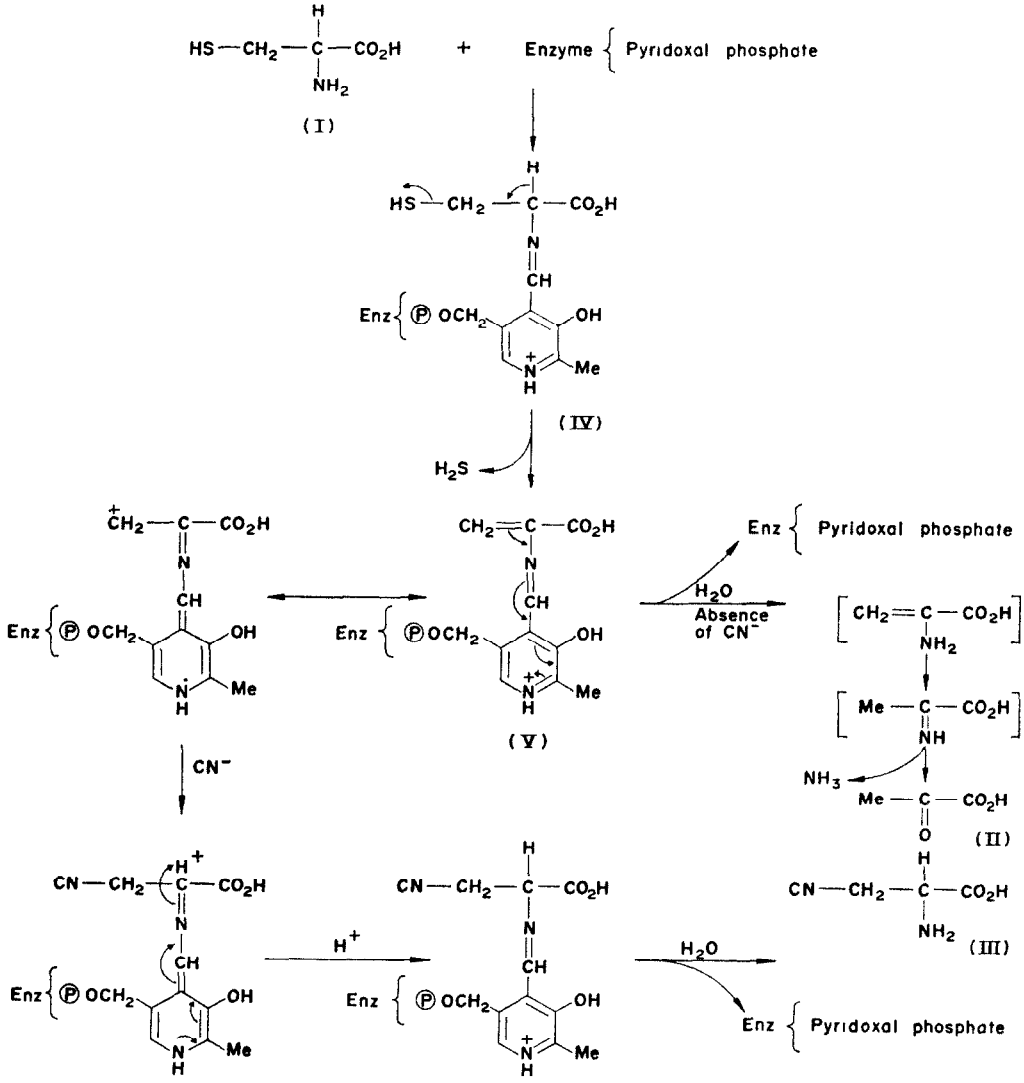
[13] SENEVIRATNE, A S and FOWDEN, L (1968) *Phytochemistry* **7**, 1039

From a consideration of the literature it would appear that β-cyanoalanine has not been described as a metabolite of *Acacia* species and indeed it may not normally be produced The *in vitro* production of β-cyanoalanine may therefore reflect the relative lack of specificity of enzymes responsible for elaborating the β-substituted alanines in *Acacia* species Such a hypothesis has been suggested in a proposed biosynthetic pathway for fluoroacetic acid in *A georginae*, via the intermediate β-fluoroalanine [7] However, the failure to incorporate fluoride in these experiments may reflect a limited specificity governed by genetic factors, since the *A georginae* seed was derived from plants devoid of fluoroacetate



EXPERIMENTAL

Enzyme preparation Seeds of *Acacia georginae* were obtained from the Northern Territory of Australia and germinated in the dark at 25–28° for 14 days The seedlings were homogenized in 10 vol cold acetone (−15) and the resulting protein ppt was filtered under vacuum, washed with acetone, and dried in a vacuum desiccator at 4° The dried acetone-powder was gently stirred for 2 hr at 4° with Tris–HCl buffer (0 1 M, pH 8 9) using 25 ml buffer per g powder After centrifugation the supernatant was dialyzed against 3 changes of 0 1 M Tris–HCl buffer pH 8 5 for 36 hr at 4° The resulting solution was used as the enzyme source

Assay procedures The complete incubation mixture contained the following components at the given final concentrations 0 1 M Tris–HCl pH 8 5, 0 5 mM L-cysteine, or *O*-acetyl-L-serine, or L-serine, 0 10 mM PALP and 0 8 mg protein, in a total vol of 2 ml The incubation mixtures containing fluoride or cyanide were 1 5 mM with respect to these substances The enzyme preparation was added to start the reaction and tubes were incubated at 30° for various times For pyruvate estimations the reaction was stopped with 0 2 ml 50% trichloroacetic acid and 0 5 ml 0 1% 2,4-dinitrophenylhydrazine in 0 2 N HCl was added After 10 min at 30° each tube was vigorously extracted with 1 ml toluene, centrifuged to separate the phases and 100 μl of the toluene supernatant containing the pyruvate-DNP was spotted on Whatman No 1 chromatography paper and developed overnight by the descending method, using *n*-BuOH–HOAc–H_2O (4 1 5, upper), as solvent The *syn*- and-*anti*-forms of pyruvate-DNP (R_f, 0 38 and 0 55) derived from each spotted aliquot were cut out, combined, and eluted by maceration in 2 ml 1 N NaOH and centrifuged The absorbance of the supernatants was read at 430 nm A standard curve was prepared in identical fashion from authentic sodium pyruvate and was linear over the range used (to 1 μmol) In some experiments pyruvate was determined by the use of LDH in which NADH consumption was determined spectrophotometrically

Sulphide estimations were carried out in Thunberg tubes, by the method of Blumenthal *et al*[3] The reaction was stopped and methylene blue was produced by mixing the incubation mixture with a solution of *N,N*-dimethyl-*p*-phenylenediamine-$FeCl_3$ reagent, contained in the side-arm

Inorganic fluoride was determined with the aid of a fluoride electrode.

Amino acid analysis by autoanalyzer was carried out on deproteinized incubations mixtures in which 20% (w/v) sulphosalicylic acid was used as protein precipitant

Protein was determined by the method of Lowry *et al*[14]

Chemicals L-Cysteine hydrochloride was purchased from Ajax Chemicals Ltd , Sydney-Melbourne, Australia, *O*-acetyl-L-serine from Calbiochem, San Diego, California, sodium pyruvate from Sigma Chemicals Ltd , St Louis, Missouri, *N,N*-dimethyl-*p*-phenylenediamine was purchased from BDH Chemicals Ltd , Poole, England Authentic β-cyanoalanine was kindly supplied by Dr F Nartey

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