Phytochemistry, 1970, Vol. 9, pp. 107 to 110. Pergamon Press. Printed in England.

THE METABOLISM OF CYCLOHEXANECARBOXYLIC ACID IN *PHASEOLUS VULGARIS*

J. G. SEVERSON, JR. and B. A. BOHM

Department of Botany, University of British Columbia, Vancouver 8, B.C., Canada

and

C. E. SEAFORTH

Division of Chemistry, University of the West Indies, Trinidad, West Indies

(*Received* 16 *June* 1969)

Abstract—Cyclohexanecarboxylic acid is converted to 1-cyclohexanecarbonyl-β-D-glucose and *N*-cyclohexanecarbonyl-L-aspartic acid when administered to leaf disks of *Phaseolus vulgaris* in the light.

INTRODUCTION

THE METABOLISM of plant growth regulators and related compounds has received attention from several laboratories. For example, indoleacetic acid,[1–4] naphthaleneacetic acid,[5] 2,4-dichlorophenoxyacetic acid,[4,6] and benzoic acid[7,8] are known to be converted to the glucose ester and/or the aspartic acid amide by a variety of plants. Our interest in the metabolism of alicyclic acids arose from current work in these laboratories on the effect of naphthenic acids[9–13] and model compounds, e.g. cyclohexane- and cyclopentanecarboxylic acids[13], on the growth and yields of certain vegetable crops. Part of this project concerned the metabolism of cyclohexanecarboxylic acid in *Phaseolus vulgaris* L., the common bush bean. To the best of our knowledge this is the first report of the metabolism of this non-naturally occurring acid by a higher plant.

RESULTS AND DISCUSSION

After 24 hr of metabolism in the presence of cyclohexanecarboxylic acid-7-^{14}C, the plant tissue was extracted and analyzed by TLC using a basic solvent system, *iso*propanol:

[1] M. H. ZENK, *Nature* **191**, 493 (1961).
[2] M. H. ZENK, *Regulateurs Naturels de la Croissance Végétale*, No. 123, Paris, France, 241 (1964).
[3] W. A. ANDREAE and N. E. GOOD, *Plant Physiol.* **30**, 380 (1955).
[4] W. A. ANDREAE and N. E. GOOD, *Plant Physiol.* **32**, 566 (1957).
[5] H. KLÄMBT, *Planta* **57**, 339 (1961).
[6] J. SUDI, *Nature* **201**, 1009 (1964).
[7] M. H. ZENK, *Planta* **58**, 668 (1962).
[8] M. H. ZENK, *Nature* **202**, 563 (1964).
[9] N. M. GLUSHKOV and A. S. YAKOVLEV, *Pchelorodstro* **6**, 25 (1963); *Chem. Abst.* **63**, 6080b (1965).
[10] A. A. ALIEV, *Zhivotnovodstvo* **25**, 25 (1963); *Chem. Abst.* **64**, 10158a (1966).
[11] M. D. POPOV, D. DIMITROV and A. STEFANOVA, *Bulgar. Akad. Nauk.* **15**, 109 (1966); *Chem. Abst.* **66**, 10095r (1967).
[12] Fr. Patent 1,433,121 (Cl. A. Oln), Esso Res. and Eng. Co. (by H. M. GUYOT); *Chem. Abst.* **65**, 17655a (1966).
[13] D. J. WORT, *Can. J. Plant Sci.*, In press.

ammonia: water (IAmW), and an acidic one, *n*-butanol: acetic acid: water (BAW). Chromatography in the IAmW system yielded four labelled spots while chromatography in BAW gave only two. Results of analysis of the spots in the IAmW system follow.

Spot No. 1

This was the largest spot observed. The R_f was 0·85 which agreed closely with that for synthetic 1-cyclohexanecarbonyl-β-D-glucose (See Table 1). This spot gave no colour with bromophenol blue, but produced a dark reaction with ammoniacal silver nitrate. Removal of this region of the chromatogram, followed by hydrolysis of the eluted material yielded only two compounds, glucose and cyclohexanecarboxylic acid. Hydrolysis of synthetic glucose ester under identical conditions yielded the same results. Synthetic ester and isolated compound also had identical R_fs in three additional solvent systems. Spot number 1 was there-

TABLE 1. CHROMATOGRAPHIC DATA

Compounds	R_fs (×100) in			
	IAmW	BuAW	IBnW	CEF*
Cyclohexanecarboxylic acid	73	92	—	—
Cyclohexanecarboxylic acid (K salt)	75	92	—	—
Benzoic acid	66	90	—	—
Glucose	39	27	—	—
Aspartic acid	08	25	—	—
Cyclohexanecarboxylic acid amide	80	85	—	—
1-Cyclohexanecarbonyl-β-D-glucose	85	78	91	94
N-Cyclohexanecarbonyl-L-aspartic acid	16	90	23	09
N-Cyclohexanecarbonylglycine	71	91	—	—

* All analyses were done using 0·25 mm (wet thickness) Cellulose MN 300 G plates. Solvent abbreviations: IAmW, isopropanol: 7% NH_4OH: H_2O (8:1:1); BAW, *n*-butanol: HOAc: H_2O (4:1:5); IBnW, isopropanol: benzene: H_2O (55:30:11); and CEF, $CHCl_3$: EtOAc: HCO_2H (35:55:10).

fore the glucose ester of cyclohexanecarboxylic acid. The colour reaction with ammoniacal silver nitrate evidently occurred as a result of some ester hydrolysis because of the alkaline nature of the reagent.

Spot No. 2

This spot was observed in the basic solvent only where it ran just behind the glucose ester. It gave no colour reaction with either bromophenol blue or ammoniacal silver nitrate, but did give a positive reaction with iodine vapour. Acid hydrolysis of eluted material yielded cyclohexanecarboxylic acid as the sole organic product. Appearance of this compound only in the ammoniacal solvent system and the hydrolysis results suggested that it might be cyclohexanecarboxylic acid amide. Comparison of eluted material with authentic amide by means of chromatography and hydrolytic behaviour supported this view. It was then found that chromatography of synthetic glucose ester in the IAmW system led to the formation of this spot in a proportion roughly equivalent to that which was observed by chromatography of the plant samples. Zenk reported similar artifact formation with indoleacetic acid.[1,2]

Spot No. 3

This was the smallest spot observed and again was seen only in the IAmW system. It gave an acidic reaction with bromophenol blue and had an R_f identical to that of free cyclohexanecarboxylic acid. Formation of this artifact by alkaline hydrolysis of the glucose ester was demonstrated using synthetic material. Since synthetic *N*-cyclohexanecarbonylglycine[14] has the same R_f as the free acid in IAmW and BuAW, eluted material was hydrolyzed with acid and examined for amino acids but none was found.

Spot No. 4

The remaining radioactive spot represented a compound present in relatively high concentration with a low mobility in the basic solvent (R_f 0·16). The spot gave a positive test with bromophenol blue and, upon hydrolysis, yielded cyclohexanecarboxylic acid and aspartic acid as the sole products. Comparison with synthetic *N*-cyclohexanecarbonylaspartic acid with regard to chromatography, colour reaction with the acid-base indicator, and products of hydrolysis showed this compound to be present.

The two radioactive spots observed in BAW had R_fs of 0·78 and 0·90. The slower moving compound was identical in all respects to authentic glucose ester, and the faster moving spot to the aspartic acid amide.

It has been established that in some animal tissues cyclohexanecarboxylic acid is readily dehydrogenated[15] and then conjugated to form hippuric acid.[16] Hydrolysis of the metabolic products from our experiments failed to yield any trace of benzoic acid. The tissues of certain higher plants have been shown[17] to hydroxylate administered benzoic acid. Either *o*- or *p*-hydroxybenzoic acid was formed, depending on the plant used, and further hydroxylation was also shown to occur. This route of metabolism of cyclohexanecarboxylic acid was discounted in bush bean by the observation that no radioactive spot was obtained which gave a positive phenolic reaction with diazotized *p*-nitroaniline.

Cyclohexanecarboxylic acid, when administered to leaf disks of *Phaseolus vulgaris*, is converted into a mixture of the glucose ester and the amide of aspartic acid. Trace quantities of other conjugates may have been formed, but were not detected by our methods. Further, our study treated only soluble conjugates. Zenk[2] has described the formation of an indoleacetic acid–protein complex by pea epicotyls. Extension of the studies of cyclohexanecarboxylic acid metabolism into this area might prove fruitful.

EXPERIMENTAL

Feeding Experiments

Leaf disks were cut from young bush bean leaves (*Phaseolus vulgaris* L. var Top Crop) using a cork borer and were floated on 6 ml of distilled water in a petri dish. To this was added 2·5 μc of cyclohexanecarboxylic acid-7-^{14}C (International Chemical and Nuclear) as the K salt. This represented a concentration of 0·02 mM. The dish was wrapped with Saran wrap to minimize water loss by evaporation and then placed in the light (5400 lux) for 24 hr at 24°. Control dishes contained the leaf disks, water, but no acid.

At the end of the metabolism period the leaf disks were washed several times with water to remove adhering labelled compound, then extracted with several changes of 80 per cent ethanol. Extraction was considered complete when the leaf disks were white. The combined ethanol extracts were evaporated to dryness, extracted with boiling water and filtered through Celite. At this point a continuous ethyl acetate extraction was attempted in early experiments. Eight hours extraction yielded only a partial separation of the major spots, spot number 1

[14] J. P. GREENSTEIN and M. WINITZ, *Chemistry of the Amino Acids*, Vol. 2, p. 892. John Wiley, New York (1961).
[15] J. B. GALPER and B. M. BABIOR, *Biochem. Biophys. Acta* **158**, 289 (1968).
[16] B. M. BABIOR and K. BLOCH, *J. Biol. Chem.* **241**, 3643 (1966).
[17] R. K. IBRAHIM and G. H. N. TOWERS, *Nature* **184**, 1803 (1959).

(the ester) being more soluble in ethyl acetate, while spot number 4 (the aspartic acid derivative) remained primarily in the aqueous phase. There was no qualitative difference in the spots observed in the two phases. All chromatography was done using thin layer plates of cellulose MN 300 G. Radioactive spots were detected with Kodak Medical X-ray film.

Synthesis of 1-*Cyclohexanecarbonyl-β-*D*-glucose*

Cyclohexanecarboxylic acid was converted into the acid chloride by refluxing the acid with a slight excess of $SOCl_2$ in dry benzene for 4 hr. After removal of benzene and excess $SOCl_2$ under reduced pressure, the residual acid chloride was dissolved in a small volume of dry pyridine and treated with an equimolar amount of β-D-glucose-2,3,4,6-tetraacetate.[18] The stoppered reaction flask was shaken to facilitate solution and allowed to stand for 3 days at room temp. The reaction mixture was poured into a mixture of 6 N HCl and crushed ice. The precipitate was collected, washed with water, then with 10% $NaHCO_3$ several times, finally with water, and then dried at room temperature. Several recrystallizations from anhydrous methanol yielded colourless crystals of 1-cyclohexanecarbonyl-2,3,4,6-tetraacetyl-β-D-glucose, m.p. 114·5–115·5°. The yield was 68 per cent.

Deacetylation was accomplished by passing a stream of anhydrous NH_3 through a methanol solution of the product from above. Excess NH_3 and solvent were removed under reduced pressure, the residue was dissolved in water, and this solution was extracted continuously with ethyl acetate for 12 hr. The ethyl acetate soluble material was purified by chromatography in BAW to yield a white product with m.p. 141–143°. A crystalline product was not obtained. This product was chromatographically homogeneous and yielded only cyclohexanecarboxylic acid and glucose on hydrolysis. Further purification was not attempted.

Synthesis of N-*Cyclohexanecarbonyl-*L*-aspartic Acid*

L-Aspartic acid (0·67 g, 0·005 M) and $NaHCO_3$ (1·43 g, 0·017 M) were stirred in 6 ml of water at room temp. Cyclohexanecarboxylic acid chloride (0·64 g, 0·005 M) was added in 5 portions over a period of 30 min. The mixture was acidified to pH 2 and continuously extracted with ether for several hours. Chromatography of the ether extract showed it to be a mixture of cyclohexanecarboxylic acid and the desired amide. Separation was done chromatographically. Elution of the desired band with methanol was followed by crystallization of the material from ethanol or ethyl acetate. This procedure yielded a white, waxy solid whose m.p. is slightly above room temperature.

Hydrolysis Procedures

Bands of unknown conjugates were scraped from the plates and eluted with methanol: water (1 : 1). The cellulose was removed by filtration and the filtrates evaporated to dryness. Band 1 (banded material corresponding to spot 1) material was hydrolyzed into its component parts by 2N NaOH for 30 min at 75°. Acidification and extraction with ether separated the acid and sugar portions of this compound.

Band 4 material was effectively hydrolyzed with 6N HCl for 30 min at 70°. The mixture was neutralized with $NaHCO_3$, evaporated to dryness, and the residue extracted with small portions of anhydrous methanol. This methanolic solution was used for chromatography. Band 2 material was also hydrolyzed this way.

Acknowledgements—This work was supported by the National Research Council of Canada to whom we express our appreciation. C. E. Seaforth was the recipient of an award from the External Aid Office of the Government of Canada. We should like to thank Professor D. J. Wort, of this Department, and Professor S. H. Zbarsky, of the Department of Biochemistry, for discussions during the course of this work.

[18] *Organic Syntheses*, Coll. Vol. III. p. 434, John Wiley, New York (1955).