THE BIOSYNTHESIS OF COUMARIN IN MELILOTUS ALBA

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The conversion of trans-cinnamic acid to coumarin in Melilotus alba has been shown to occur by way of o-coumaric acid, o-coumaric acid β-glucoside and coumarinic acid β-glucoside (Stoker and Bellis, 1962; Kosuge and Conn, 1961). The partial conversion of o-coumaric acid β-glucoside to the cis isomer, coumarinic acid β-glucoside, on exposure of plant extracts to artificial light led Haskins and Gorz (1961) to suggest that the biosynthetic isomerization might be non-enzymatic. The experimental work described in this report was designed to investigate this biochemical isomerization and to demonstrate, if possible, the existence of an isomerase enzyme system in Melilotus alba plants.

Trans-cinnamic acid - 3-C^{14} was synthesised from benzaldehyde-1-C^{14} and malonic acid according to the method of Brown and Neish (1955). The acid was dissolved in the calculated quantity of 5% sodium bicarbonate solution. Shoots, approximately 6 inches in height, were cut from plants of Melilotus alba and the stems trimmed under water. The cut ends of the shoots were immersed in the aqueous cinnamate solutions (0.5 ml. of solution, approximately 0.068 M. per 4 g. fresh weight). In one experiment the shoots were subject to daylight illumination for 6 hours, while other shoots were kept in the dark for 6 hours. At the conclusion of the metabolic period the free coumarin and coumarin

aglycone were isolated and the total coumarin assayed quantitatively and for radioactivity as described earlier (Stoker and Bellis, 1962).

The results of these experiments, which are shown in Table I, demonstrate that there is appreciable conversion of trans-cinnamic acid to coumarin by shoots of M. alba kept in the dark. This indicates the presence of an isomerase in the plants.

Table I. The conversion of trans-cinnamic acid-3-C^{14} to coumarin in Melilotus alba shoots.

	Total coumarin	Specific activity
Shoots exposed to daylight	10.03 mg.	1.01×10^5 c.p.m/mM.
Shoots kept in the dark	9.97 mg.	0.66×10^5 c.p.m/mM.

The specific activity of the trans-cinnamic acid-3-C^{14} was 2.219×10^7 c.p.m/mM.

The existence of an isomerase enzyme system in M. alba plants was further confirmed by the preparation of an aqueous plant extract with the ability to convert o-coumaric acid β-glucoside into coumarin.

Fresh plant material of M. alba, 50 g, was frozen with liquid nitrogen, ground to a fine powder and then mixed with 50 ml. of distilled water. After standing at 25°C for 24 hours the suspension was strained through muslin and the filtrate centrifuged. The supernatant was continuously extracted with ether for 48 hours to remove coumarin and other ether soluble materials.

Aliquots, 2 ml., of this aqueous extract were incubated at 25°C for 6 hours in the dark with 0.2 ml. of an approximately 0.05 M. solution of the β-glucoside of o-coumaric acid-2-C^{14} in 5% sodium bicarbonate solution. The β-glucoside of o-coumaric acid-2-C^{14} was prepared from helien and malonic acid-2-C^{14} according to the method of

Helferich and Lutzmann (1939). At the conclusion of the incubation period the free coumarin and coumarin aglycone were isolated and aliquots of an ether solution of the bulked total coumarin subject to chromatography on Whatman No. 1 paper using several solvent systems. The solvent systems used were - (i) benzene-acetic acid-water (1:1:2), (ii) n-propanol-ammonia (7:3) and (iii) ethyl methyl ketone-water-diethylamine (921:77:2). The developed chromatograms were scanned for radioactivity with a G.E.C. thin end window Geiger-Müller counter. Coumarin was detected by its fluorescence after spraying with sodium hydroxide solution. The coumarin spot on each chromatogram corresponded in position to a centre of high radioactivity.

When the above technique was applied to samples of plant extract previously heated at 98 - 100°C for 30 minutes the spots corresponding in position to coumarin showed no measurable radioactivity.

Discussion

The conversion of trans-cinnamic acid to coumarin by Melilotus alba plants kept in the dark indicates that the plants do contain an isomerase enzyme system. This has been confirmed by the preparation of a heat labile plant extract which is able to catalyse the isomerization of o-coumaric acid β-glucoside to coumarin. Further studies on this enzyme are in progress.

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