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SHORT COMMUNICATION

CONCERNING THE MECHANISM AND CONTROL OF α-AMYRIN BIOSYNTHESIS*

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Abstract—Experiments have shown that the variation in the ratios of α- to β-amyrin which are found in plants is not controlled by *S*-adenosylmethionine. The radioactive compound produced in peas after feeding with radioactive methionine is 24-methylene-lanosterol.

THE MECHANISM which has been postulated[1] for the conversion of squalene, via the epoxide,[2] to α-amyrin is the same as that[1] for the formation of β-amyrin, except that in the former case a 1,2-methyl migration occurs. In both cases the mechanism involves the same steric arrangement of the carbon atoms in a concerted process, and hence the controlling element in the choice of whether α- or β-amyrin is produced should reside in the intrinsic chemistry of the folded squalene molecule at the moment of reaction on the enzyme. This would suggest that the ratio of α- to β-amyrin formed would always be the same, but such does not appear to be the case. In some plants, only α-amyrin is reported,[3–6] in others only β-amyrin,[7–9] in still others both are present with either α-amyrin[10] or β-amyrin[11] predominating. While these observations could be due to selective metabolism, there is a strong suggestion that selective biosynthesis is taking place. One possible way in which the biosynthesis could be controlled is through a cofactor requirement. The 1,2-migration might arise by an addition-elimination process in which *S*-adenosylmethionine serves as an acceptor-donor of the methyl group.

Germinating peas are known[12,13] to form both β-amyrin and a minor triterpenoid

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identified in this laboratory as α-amyrin by its GLC retention time* and more thoroughly by Goodwin *et al.*[11] In earlier work Castle, Blondin and Nes[14] observed that methyl labelled methionine led to a labelled substance with essentially the same GLC retention time as the peak identified as α-amyrin. This work has been repeated, and we should like to report that further purification of this labelled substance by TLC completely separated it from α-amyrin. The metabolite suspected of being α-amyrin had the same R_f and the same gas-liquid chromatographic retention time as 24-methylenelanosterol, acidic and hydroxylic derivatives of which are well described in the plant kingdom.[15] Consequently, the control of 1,2-migration does not appear to reside in *S*-adenosylmethionine.

EXPERIMENTAL

Methods and Materials

Authentic α- and β-amyrin were obtained from Pierce Chemical. Their GLC retention times relative to cholesterol, 1·78 and 1·58, respectively, were identical with those of the minor and major components, respectively, of the triterpenoid fraction from peas. The chromatography was carried out on XE-60 (Analabs) deposited on silanized chromasorb-W (Analabs).

Radioactive counting was carried out on a planchette with a thin window Geiger (Nuclear-Chicago Model C115 automatic, low background counter). TLC plates were scanned for activity using a radiochromatogram scanner (Atomic Accessories, Inc. Model RSC-363).

Alumina for liquid-solid chromatography was Woelm, "neutral," activity grade 1, deactivated with 2 per cent of water. The eluant was a graded system of diethyl ether into hexane. TLC was carried out on 0·25 mm plates of silver nitrate impregnated silica gel G (E. Merck). The plates were prepared from a slurry made by shaking 30 g of silica gel G with a silver nitrate solution (60 ml of water and 13 g of silver nitrate).

Incubation

30 dry seeds of *Pisum sativum* (Burpee's Blue Bantam), treated with Spergon to inhibit microbial growth, were placed in a small dish containing $1{\cdot}97 \times 10^6$ cpm of L-methionine-methyl-C^{14} (New England Nuclear Corp., 1·00 mC=11·9 mg), 99·43 mg of the dibenzylethylenediamine salt of DL-mevalonic acid (California Corporation for Biochemical Research), and 2 0 ml of distilled water. All the seeds were in contact with the solution, but none were completely covered. The seeds were allowed to germinate for 3 days at room temperature (23–28°). Distilled water was added periodically to maintain the level.

At the end of the germination period, the seeds were washed with distilled water and ground in acetone with a mortar and pestle. A total of $1{\cdot}83 \times 10^6$ cpm was taken up by the peas. The ground seeds were extracted with refluxing acetone in a Soxhlet for 17 hr.

The residue from the acetone extract was partitioned between 150 ml of ether and 150 ml of water, and the aqueous phase was re-extracted with two 50-ml portions of ether. The total ether layer was then washed with 50 ml of water. The residue from the ether extract was hydrolyzed for 1 hr in 25 ml of refluxing 10 per cent ethanolic KOH. After removal of most of the ethanol under reduced pressure, the residue was partitioned between 100 ml of water and 100 ml of hexane, and the water layer was re-extracted with three 50-ml portions of hexane. The total hexane layer was washed with two 50-ml portions of water and evaporated to dryness.

The residue from the hexane extract was redissolved in a small volume of hexane and chromatographed on 7·0 g of 2 per cent deactivated alumina in a 1·0-cm (i.d.) column. The column was eluted with the following solvents in 3·0-ml fractions: fractions 1–10 (30 per cent ether in hexane), 11–40 (50 per cent ether in hexane), 41–54 (100 per cent ether), 55–60 (ethanol). An aliquot of each fraction was removed and counted. A plot of radioactivity versus sample number gave four regions of activity. These were fractions 15–20, 27–32, 42–47, and 56–59. The first three peaks corresponded to positions for polycyclic isopentenoids with two, one, and no methyl groups at position C-4 of ring A. α- and β-amyrin were found in the first peak.[12,14]

Fractions 15–20 (Peak 1) were combined. The acetates of this combined fraction were dissolved in 1·0 ml of CH_2Cl_2 and aliquots examined on $AgNO_3$-impregnated TLC.[16] Four different chromatograms were run using differing amounts of the radioactive material. In each case, a mixture of α-amyrin acetate and lanosterol

* This was first shown by R. T. van Aller and W. R. Nes (unpublished observations). The identification has been repeated by the present authors and is included in the experimental section.

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acetate was used as a standard. After development of the plates in $CHCl_3$, hexane and acetic acid (50:150:1) they were sprayed with a 0·1 per cent 2′, 7′-dichlorofluorescein in ethanol and examined under u.v. light. Radioactivity was determined using the radiochromatogram scanner. Typical R_f values are: β-amyrin 0·41, α-amyrin 0·41, lanosterol 0·25. Radioactive spots had R_f's of 0·16 and 0. In some cases the plates were redeveloped to give further movement of the samples. In every case, the radioactivity appeared as two peaks of similar magnitude, one at the origin and one moving slower than lanosterol acetate. The R_f of 24-methylenelanosteryl acetate (prepared via the Wittig reaction by Dr. J. P. John) was the same as that of the faster moving radioactive metabolite. The mixed metabolites also have already been found[14] to have a peak in gas–liquid chromatography with a relative retention time (cholesterol as standard) which is the same as α-amyrin–(1·78), but we find[1] that 24-methylenelanosterol has a nearly identical retention time (1·72).