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FORMATION OF A Δ^9-DODECENOIC DIBASIC ACID FROM LINOLEIC ACID BY YOUNG PEA LEAVES

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Abstract—A "crude plastid" preparation from young pea leaves converts linoleate [1-^{14}C] or [U-^{14}C] (ammonium salt or free acid) into a C_{12} dibasic acid with a double bond at Δ^9.

INTRODUCTION

Linolenic acid ($C_{18:3}$) is formed in young leaves by direct desaturation of linoleic acid ($C_{18:2}$), as shown by the direct conversion *in vivo* of linoleic acid [^{14}C] [1] and recent works of Slack and Roughan [2], and of Richards and Quackenbush [3] are consistent with these views. A second biosynthetic pathway has been proposed by Stumpf for the linolenic acid of spinach chloroplasts involving the desaturation and subsequent elongation of a C_{12} precursor to hexadecatrienoic acid ($C_{16:3}$) and linolenic acid [4]. *In vitro* experiments with subcellular fractions of green leaves are now necessary to examine the biosynthetic pathway of linolenic acid in more detail.

In 1974, Tremolières and Mazliak [1] described the conversion of linoleate [U-^{14}C] (ammonium salt) into α-linolenic acid by a crude plastid preparation of young pea leaves. The acid was identified by its GLC, R_t and by TLC on $AgNO_3$ impregnated silica gel. However, in subsequent analyses we observed that, in other chromatographic systems, or after catalytic hydrogenation, the behaviour of the radioactive product was different from that of authentic α-linolenic acid. In this paper we describe the identification of the product formed *in vitro* from linoleate [1-^{14}C] or [U-^{14}C] by the "crude plastid" fraction. Because the amounts of the product obtained were largely below the μg level, it was not easy to use conventional methods of identification and only analytical methods coupled with simultaneous detection of the radioactivity could be utilised.

RESULTS AND DISCUSSION

The idea that the product obtained from the plastid fraction was not simply linolenate arose when we found that it was not soluble in chloroform but instead remained in the aqueous–methanolic phase during lipid extraction of the incubation medium using the method of Bligh and Dyer [5]. After direct saponification and transmethylation of the incubation medium, the total fatty acid Me esters were analysed by $AgNO_3$–Si gel TLC. The spot corresponding to triunsatured fatty acid Me esters was removed, the compounds recovered and analyzed by Si gel TLC. We observed that the radioactive product migrated below the un-labelled triunsaturated fatty acid Me esters (which were pure methyl α-linolenate, as shown by MS and IR analysis). The radioactive spot was then removed from the plate and eluted.

Further TLC analysis showed that the R_f value of the compound on Si gel corresponded with saturated dibasic acid Me esters but the lower R_f value on $AgNO_3$–Si gel suggested that it was an unsaturated dibasic acid. Unsaturation was confirmed by the change of GLC R_f after catalytic hydrogenation and GLC analysis on BDS, DEGS and SE30 stationary phases using authentic dibasic acid Me esters showed the hydrogenated product to be dimethyl dodecane-1,12-dioate. The difference in R_t between the non-hydrogenated and hydrogenated product was compatible with a compound containing one double bond. Oxidative cleavage with $KMnO_4$ [6] gave only one radioactive fragment which was identified as nonane-1,9-dioic acid showing that the double bond in the radioactive compound was located between C-9 and C-10. Thus, the product formed by our plastid preparation is dodec-9-ene-1,12-dioic acid.

Linoleic acid (ammonium salt or as free acid) was the only fatty acid of ten acids tested which could be converted into the dicarboxylic acid by our plastid preparation. No added cofactors were necessary, but the reaction was completely inhibited if the preparation was boiled beforehand or by anaerobiosis. The crude plastid fraction was more active than crude mitochondrial or microsomal fractions but a plastid fraction purified on a discontinuous sucrose gradient remained active. If plastids were osmotically disrupted they lost their ability to transform linoleate to the $C_{12}\Delta^9$ dibasic acid.

The optimum pH for the conversion was between 8 and 8.5. This pH differs markedly from the acidic optimum usually found for lipoxygenase which is the usual degradative enzyme for linoleic acid [7]. The reaction was also strongly dependent on the age of the leaves and the *in vitro* formation of the dibasic acid closely followed the *in vivo* production of α-linolenic acid from linoleate in the whole leaf. This reaction occurred in darkness or in light, and also with a plastid preparation from etiolated leaves showing that the observed transformation of linoleic acid was not a photo-oxidation. We

were not able to observe the reaction with plastid preparations from adult leaves which synthesize only small amounts of α-linolenic acid [8].

The significance of the conversion of linoleate [1-^{14}C] or [U-^{14}C] into an unsaturated C_{12} dibasic acid remains somewhat obscure. Nevertheless the parallelism between the biosynthesis of α-linolenic acid *in vivo* and the activity of conversion of linoleate *in vitro* is intriguing especially since we found that the reaction only took place in tissues which synthesized α-linolenic acid in great amounts. We should like to suggest that in our *in vitro* system the desaturating enzyme acting specifically on linoleic acid released into the incubation medium, for unknown reasons, a highly unstable di- or tri-unsaturated intermediate which was immediately oxidized and cleaved on the double bond nearest the point of maximum instability. This process would most probably give rise to the observed $C_{12}\Delta^9$ dibasic acid.

EXPERIMENTAL

Pisum sativum (var. Alaska) 7 days old, grown in short days conditions (or in complete darkness for the study on etiolated leaves), were placed under continuous light (16700 lx, 25°) for 24 hr or maintained in the dark. Young leaves (20 g) were gently ground in a mortar in the following medium: sucrose 0.4 M, BSA 0.1%, KCl 10 mM, $MgCl_2$ 10 mM, cysteine 4 mM, in Tris–HCl buffer 0.1 M, pH 7.4. Homogenate was filtered through 2 sheets of miracloth and the filtrate centrifuged at 500 *g*. The first pellet was discarded and the supernatant centrifuged at 3000 *g* for 15 min. The second pellet was named "crude plastid fraction" or "3000 *g* fraction". This fraction has been occasionally purified in a discontinuous sucrose gradient consisting of 5 ml of 2 M sucrose at the bottom of the tube, 10 ml 1 M sucrose above this band and the crude plastid fraction in 10 ml 0.4 M sucrose at the top. The tube was centrifuged at 1000 *g* for 20 min; the band just above the 2 M sucrose soln contains a high proportion of intact plastids and few other cellular contaminations as judged by phase-contrast microscopy. Crude or purified fractions were suspended in the isolation medium and gently homogenized in a Potter apparatus with a Teflon pestle. The homogenate was incubated at room temp. with linoleic acid [1-^{14}C] (NEN Germany 2.7 nM per incubation tube, sp act 0.05 mCi per mol). The precursor was administrated as NH_4 salt prepared by heating linoleic acid with 0.2 ml of NH_4OH (4.4% in H_2O). The total incubation medium (without lipid extraction) was saponified with 5 ml of 0.5 M methanolic NaOH for 15 min at 65°, the fatty acids methylated with 4 ml of BF_3–MeOH for 5 min. at 65° and the Me esters extracted in 10 ml of pentane. GC–RC was performed using different chromatographic systems (a) butanediol succinate (BDS) (20% on Chromosorb W at 190°) (b) SE 30 (20% on Chromosorb W at 230°). The columns were 3 m long and 6 mm i.d. Two TLC systems were used (a) Si gel G with petrol–Et_2O–HOAC, (70:30:0.4) [9], (b) Si gel G impregnated with $AgNO_3$ [10]. The position of the double bond was determined by periodate–permanganate oxidation according to ref. [6].

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