Phytochemistry, 1970, Vol. 9, pp. 1997 to 2002. Pergamon Press. Printed in England.

LIPID BIOSYNTHESIS IN SENESCING COTYLEDONS OF CUCUMBER

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(*Received* 12 *December* 1969)

Abstract—Cotyledons of cucumber, excised from the plant and floated on buffered solutions, possess the ability to incorporate ^{14}C-acetate into five major lipid fractions. Maximum observed rates of incorporation were into phosphatidyl choline and neutral lipid. Senescing cotyledons progressively lose the ability to incorporate ^{14}C-acetate into lipid. Decreased rates were first observed for phosphatidyl ethanolamine and later for other lipid classes including the galactolipids.

INTRODUCTION

THE YELLOWING of cotyledons of cucumber (*Cucumis sativus* L.) is accompanied by a rapid loss of total protein, RNA[1] and lipid.[2] In the case of those substances subject to turnover (that is, continual breakdown and resynthesis) there are two ways in which the overall level of the substance could be disturbed. Thus a decrease in level could be due to either an increased rate of breakdown or to a decline in biosynthesis. Plant proteins appear to be subject to turnover[3,4] and the loss of protein during leaf ageing can be accounted for by decreased synthetic activity. This has been demonstrated for both leaves allowed to yellow in the light[5] and in the dark.[6,7]

The major lipids of green tissue are the phospho- and glycolipids. There is evidence that both these types of lipid are subject to rapid turnover in the plant cell. In the case of phospholipids this evidence arises from pulse labelling studies[8] and from the widespread occurrence of various phospholipases.[9] The turnover of glycolipids also appears to occur rapidly in plant tissue.[10,11] Enzymes involved in breakdown have been reported in leaves[12] and

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chloroplast preparations.[13] The biosynthetic process has been demonstrated by labelling studies with barley.[14,15]

The present experiments were carried out in order to investigate the ability of ageing tissue to synthesize polar and neutral lipids. The incorporation of labelled acetate into five major lipid fractions was studied for tissue of various ages. In addition, measurements of the total uptake of precursor were made for tissue at three developmental stages. The rate of photosynthesis was also investigated. This was in order to see if the observed decreases in acetate incorporation occurred at the same stage of senescence as did the decline in photosynthetic activity.

RESULTS

The rate of incorporation of acetate-2-^{14}C was used as a measure of biosynthetic activity. In a preliminary experiment the effects of light and dark and the period of labelling were investigated. Cotyledons were excised from the plant and floated in Petri dishes containing a solution of the labelled precursor. After incubation the tissue was extracted with a $CHCl_3$–MeOH mixture and aliquots of the extract were taken for scintillation counting. The extract was then freed of water-soluble impurities and chloroform-soluble incorporation was determined. With incubation periods of 2, 19 and 24 hr the uptake of acetate-2-^{14}C into the $CHCl_3$–MeOH extract was higher when illumination was provided than when cotyledons were maintained in the dark (Table 1). This also applied to chloroform-soluble incorporation.

TABLE 1. INCORPORATION OF ACETATE-2-^{14}C IN THE LIGHT AND IN THE DARK

Incubation	Activity counts/min/cotyledon	
	Total solvent-soluble uptake	Total chloroform-soluble incorporation
(A) 2 hr dark	5040	1970
light	6700	3320
(B) 19 hr dark	9310	4980
light	38,240	15,110
(C) 24 hr dark	14,290	7980
light	34,960	13,880

Green tissue was used and the incubation solutions contained 10 μc of acetate per 20 ml.

tion. The incorporation of acetate into substances soluble in chloroform was greatest for an incubation period of 19 hr. As a result of these observations it was assumed that incorporation during the 2-hr period used in further experiments reflected the ability to synthesize lipid rather than the amount of lipid present.

Cotyledons were incubated in the light for 2 hr and then disrupted in the solvent mixture. A chloroform solution was obtained and 10-μl aliquots used for TLC. After development the various lipids were localized with iodine and incorporation into the five major fractions was determined. This procedure was carried out with batches of cotyledons of various ages. In

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this way it was hoped to investigate the pattern of biosynthesis during growth and senescence. With tissue aged 7 days the fraction containing the highest number of counts was the NL fraction (Fig. 1). During the next 3 days the main change was a very large increase in labelling of

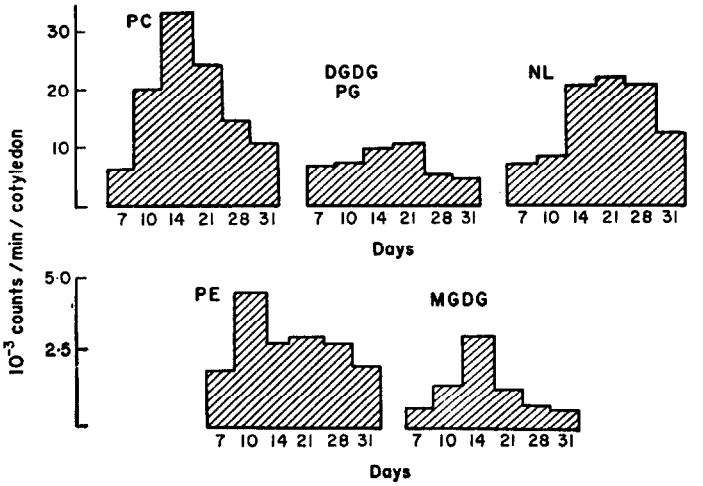

FIG. 1. INCORPORATION OF ACETATE-2-^{14}C INTO VARIOUS LIPID FRACTIONS.
Using incubation solutions containing 50 μc of acetate/20 ml, experiments were carried out with cotyledons of different ages. PC.

Abbreviations used

PC, Phosphatidyl choline; PE, Phosphatidyl ethanolamine; PG, Phosphatidyl glycerol; DGDG, Digalactosyl diglyceride; MGDG, Monogalactosyl diglyceride; NL, Neutral lipid.

the PC fraction. This amounted to over a three-fold increase. There was also a large increase in the rate of incorporation into the next major phospholipid, PE. These changes accompanied the phase of expansion of the lamina. During the period 14–21 days maximal rates were observed for incorporation into all classes of lipid except PE. During the period of yellowing, from 21 to 31 days, incorporation into each fraction decreased rapidly. At 28 and 31 days labelling was most extensive for the neutral lipid fraction. Throughout the experiment labelling into the fraction containing DGDG was much more rapid than into MGDG.

Although changes in incorporation were observed it was necessary to ensure that this was not merely due to a varying permeability of the tissue at different stages of growth and senescence. Measurements of total soluble uptake showed that ageing tissue becomes progressively more permeable to acetate (Table 2). Thus the decreased incorporation observed with older tissue was not due to a decreased uptake of precursor. On the contrary, the highest level of uptake was with yellow tissue.

As the formation of fatty acids can involve a requirement for active photophosphorylation[16] it is possible that the photosynthetic activity of the tissue is a factor in determining the rate of lipid biosynthesis. For this reason measurements of photosynthesis were carried out with attached cotyledons of various ages. The highest observed rate was at 19 days. During the next 5 days there was a 70 per cent decrease in the rate (Fig. 2).

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TABLE 2. TOTAL CHLOROFORM–METHANOL SOLUBLE UPTAKE

Tissue	Counts/min/cotyledon × 10^{-3}
(a) Green; unexpanded	18·8
(b) Green; expanded	41·0
(c) Yellow	74·0

Measurements were made on cotyledons of various ages using incubation solutions containing 50 μc of acetate per 20 ml. The results are expressed as average values for each of three developmental stages. Each value is the mean of two results.

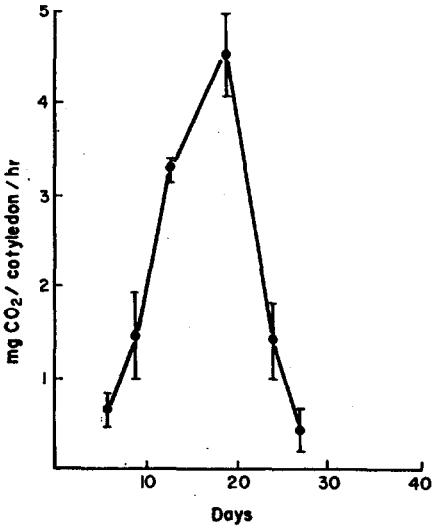

FIG. 2. PHOTOSYNTHESIS.
Replicate determinations were made on attached cotyledons of various ages. Vertical lines drawn through each point represent the standard error.

DISCUSSION

The senescence of cucumber cotyledons is accompanied initially by a breakdown of galactolipids and at a later stage by a loss of phospholipid.[2] The present experiments were undertaken to see if this decrease in lipid content could be accounted for by a decreased rate of biosynthesis. The uptake of labelled precursor was measured by counting aliquots of a $CHCl_3$–MeOH extract. This gave a measure of total soluble uptake including unincorporated ^{14}C-acetate. Incorporation into a water-washed chloroform extract was taken as an approximate measure of incorporation into lipid. Both chloroform-soluble and total soluble uptake were increased if the incubation period was lengthened from 2 to 19 hr, or if illumination was provided at an intensity of 8000 lx (Table 1). The fact that a further increase in the period of incubation from 19 to 24 hr led to no further increase in incorporation in the light led to the adoption of the short 2-hr incubation period as standard. This was to avoid the possibility of saturation of labelling. Tissue was sampled at various stages of development and senescence. Samples included green unexpanded cotyledons (7 days), expanded but non-senescent tissue

(14 days) and yellow (28 days) and yellow-brown (31 days) senescent tissue. Older tissue was found to take up label more rapidly than younger tissue. This probably reflects the increase in permeability which occurs during senescence.[17,18] In spite of the high total uptake observed with yellowing tissue the incorporation of acetate into each of five lipid fractions was found to decrease as senescence advanced (Fig. 1). The fractions examined represent the major lipids of cucumber cotyledons and the results may be compared with previously reported changes in relative overall amounts.[2] The observed decrease in incorporation could be due to a breakdown of the biosynthetic machinery, a change in the size of the metabolic pool of acetate or to a lack of precursor material. Alternatively the limiting factor could be the supply of biochemical energy. With these possibilities in mind measurements were made of the rate of photosynthesis in tissue of various ages. A decrease in rate might be expected to reduce the availability of carbon skeletons and also of ATP energy from photophosphorylation. Using attached cotyledons it was found that the decline in photosynthesis coincided with the decrease of ^{14}C-acetate incorporation into the DGDG + PG and NL fractions (compare Figs. 1 and 2). Decreases of incorporation for the other fractions were observed at an earlier stage. Thus the observed decline in photosynthesis does not appear to control the early decrease in biosynthesis of, for example, PE.

EXPERIMENTAL

Plant Material

Plants of cucumber (*Cucumis sativus* L.) variety Long Green Trailing were raised as described previously.[2] The age of cotyledons was taken as the time from sowing.

Incorporation of Acetate-2-^{14}C

Batches of eight cotyledons were washed (H_2O) and floated in petri dishes containing 20 ml of 0·05 M phosphate buffer, pH 5·0, and 1–50 μc of labelled NaOAc. Buffer of low pH was used in order to facilitate the uptake of acetate as the uncharged form. The incubation period varied between 2 and 24 hr. Illumination was provided at an intensity of 8000 lx (Hg fluorescent lamp) although in some experiments the dishes were covered with Al foil in order to measure the rate of incorporation in the dark. After incubation, batches of cotyledons were washed for 3 min in 2 × 1 H_2O and then disrupted in 40 ml of 2:1 $CHCl_3$–MeOH using an overhead grinder (full speed for 60 sec). The homogenate was transferred to a conical flask and the grinding vessel washed with 10 ml of solvent. Insoluble material was removed by filtration and the filter paper rinsed with an additional 10 ml of solvent. Aliquots of the filtrate were taken for scintillation counting. These samples gave an estimate of total $CHCl_3$–MeOH soluble uptake. This includes acetate taken up but not metabolized. To the remainder of the filtrate was added 10 ml of 0·1 M sodium acetate (unlabelled). The aqueous upper phase which separates out was removed by suction. Aliquots of the lower phase were taken for scintillation counting or for lipid fractionation by TLC.

Lipid Fractionation

The only available method of separation with the required resolution was found to be TLC . Although column separations on silic acid have been described by other workers,[19] trial experiments in which the eluates were monitored by TLC indicated that a certain amount of cross-contamination occurs.

Conventional TLC on glass plates results in a series of spots spread along the plate. To elute a particular fraction the relevant area of silica gel must be scraped from the plate. Even if the plate is initially wetted with solvent it is difficult to avoid some loss of silica gel as airborne dust. For this reason separations were carried out using prepared thin layers of silica gel on plastic backing sheet (Eastman Chromagram Sheet 6061: Kodak Ltd., Research Chemical Sales Division, Kirkby, Liverpool). The solvent system was $CHCl_3$–MeOH–HOAc–H_2O (85:15:10:3·5). After development the separate spots were visualized with I_2 vapour. The pattern of separation was as described elsewhere.[3] The various constituents were identified by chromatographic mobility and, in the case of PC, PE and NL, by comparison with standards. Ninhydrin spray was used to confirm the position of PE. The solvent system used did not resolve DGDG and PG. The presence of two components at

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this point on the chromatogram was demonstrated by the separation of acidic and non-acidic lipids prior to TLC.[20] The plastic backing sheet was cut with scissors into areas representing the various major lipid fractions. Each square of silica gel coated plastic was placed in a vial containing a 3-ml aliquot of scintillation fluid.[8] The silica gel coating was dislodged in the scintillation liquid by prodding with a glass rod. This ensured uniform geometry of counting for different samples. Duplicate samples were counted for an appropriate period using a Packard Tri Carb 200 sample automatic change, liquid scintillation spectrometer.

Infra-red Gas Analysis

Measurements of CO_2 uptake were made on single cotyledons attached to the plant and enclosed in perspex flow chambers. Two types of chamber were used according to the size of the cotyledon. The gas-flow inlet and outlet were at opposed positions with each type. In addition a gas-mixing device was employed in the region of the inlet. In the case of the larger chamber this consisted of an arrangement of thin plastic baffles while with the smaller chamber the air flow was restricted by passage through a perforated plastic base plate. This ensured a slight positive pressure at the inlet end of the chamber resulting in a turbulent ingress of air. These measures were designed to ensure thorough mixing of the supply of atmospheric air to the cotyledon. Differences in CO_2 content of the supply and output gas were measured by i.r. gas absorption using a Type L/SCR instrument. A flow rate of 104 ml/min was used for the majority of the experiments. Replicate measurements were made using a series of cotyledons from the same population. With each cotyledon measurements were first made using a light intensity of 5000 lx. The lights were then turned off and additional measurements were made immediately. The rate of CO_2 output in the dark was added to the rate of uptake in the light. This was taken as a measure of photosynthesis. All measurements were carried out with plants maintained at 25°.

Acknowledgement—Financial support from the Agricultural Research Council is gratefully acknowledged.

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