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PROTEIN SYNTHESIS BY DISKS OF PUMPKIN MESOCARP

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Abstract—Marked synthesis of protein occurred when freshly prepared disks (1 × 10 mm) of mesocarp of ripe pumpkin (*Cucurbita pepo* L.) were incubated for 48 hr at 26° under moist conditions. The incubation of the disks was accompanied by an incorporation of [^{14}C]-leucine into protein and by a 50 per cent increase in the amount of insoluble nitrogen. The intracellular distribution of [^{14}C]-leucine incorporated into protein indicates that excision and incubation led to a steady synthesis of protein in each of the major cellular fractions.

INTRODUCTION

INCUBATION in aerated distilled water causes marked changes in the metabolism of freshly prepared thin disks of a wide variety of plant organs and tissues.[1,2] In this paper treatment of disks of tissue in the above manner is called ageing. Previous work[2] suggested that ageing of disks of pumpkin mesocarp was accompanied by protein synthesis. The possibility that relatively senescent tissue in fruits responds to excision by synthesizing appreciable amounts of protein is relevant to our understanding of the control of protein synthesis, the role of protein synthesis in fruit ripening, and the interpretation of experiments with aged disks in general. This paper reports experiments designed to see whether ageing of disks of mesocarp of ripe pumpkin resulted in protein synthesis.

Two complementary experimental approaches were used. These were the assessment of the effect of ageing on the total protein content of the disks, and the measurement of [^{14}C]-leucine incorporation into protein during ageing. Particular attention was paid to the following points in the design of the experiments. First, the evidence that well-washed preparations of cell walls may contain protein[3] was taken into account. Second, the assay of total protein in plant cells depends largely upon the adequacy of the method used of separate protein from non-protein nitrogen.[4] Consequently several methods of separating the two forms of nitrogen were compared. Finally, it is vital to bear in mind that the incorporation of radioactivity from [^{14}C]-leucine into a protein fraction is proof of protein synthesis only when it has been established that the incorporation represents the inclusion of [^{14}C]-leucine into peptide linkages throughout protein molecules.[5] Thus I took care to characterize the incorporation of label from [^{14}C]-leucine into the protein fractions.

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RESULTS

Estimates of Total Protein

The procedure used to extract protein gave a residual fraction that contained appreciable quantities of nitrogen (Table 1). This residue is called the cell-wall fraction. In both fresh and aged disks 35–45 per cent of the total insoluble nitrogen remained associated with the cell-wall fraction even after repeated extraction. The probability that at least some of the nitrogen in the cell-wall fraction was protein was borne in mind in all subsequent experiments.

Five methods of separating protein from non-protein nitrogen in extracts of both fresh and aged disks were compared (Table 1). The dialysis residues and the precipitates obtained

TABLE 1. EFFECT OF AGEING FOR 72 hr ON INSOLUBLE NITROGEN CONTENT OF DISKS OF PUMPKIN MESOCARP*

Cell fraction	Method of separating protein from cell extract	Insoluble nitrogen (mg/10 g initial fresh wt.)	
		Fresh disks	Aged disks
Cell extract	Dialysis	3·44	5·17
	Trichloroacetic acid (5%) at 2°	3·78	5·97
	Trichloroacetic acid (2·5%) at 98°	3·10	5·22
	Boiling ethanol	3·84	7·03
	Boiling water	4·14	4·25
Cell wall		2·03	2·86

* All the data refer to a single batch of disks. Half of the freshly cut disks were extracted at once, and the rest were extracted after ageing. Replicate samples of each extract were taken for comparison of the methods for isolation of protein.

with both hot and cold trichloroacetic acid contained roughly similar amounts of nitrogen. This similarity was obtained consistently with extracts of both fresh and aged disks. It is likely that most of the nitrogen in the dialysis residues and the precipitates obtained with trichloroacetic acid was protein. This view is strengthened by independent assay, by the Biuret method, of the protein content of the precipitates obtained with cold trichloroacetic acid (Table 2). The use of boiling water or boiling ethanol to separate protein and non-protein nitrogen was unsatisfactory. The nitrogen content of the precipitates varied considerably and showed no consistent relationship to the values obtained by dialysis and by precipitation with trichloroacetic acid.

The data in Table 1 show that ageing caused a marked increase in the amount of insoluble nitrogen that could be extracted from pumpkin disks. This increase was paralleled by an increase in the amount of extractable material insoluble in trichloroacetic acid and which gave the Biuret reaction (Table 2). The nitrogen content of the cell-wall fraction also showed a significant increase during ageing. Most of the increase in insoluble nitrogen took place in the first 24 hr of ageing (Table 3).

TABLE 2. ASSAY OF PROTEIN IN EXTRACTS OF FRESH AND 72-hr AGED DISKS OF PUMPKIN MESOCARP

Method of assay*	Protein (mg/10 g initial fresh wt.)	
	Fresh disks	Aged disks
Biuret reaction	26·3	41·5
Kjeldahl determination of total nitrogen	22·7†	36·0†

* Replicate samples of cell extracts were made 5% with respect to trichloroacetic acid at 2°. The protein content of the resulting precipitates was assayed as shown. Each figure represents the mean of three samples.

† Values for total nitrogen multiplied by 6·0.

TABLE 3. EFFECT OF AGEING FOR 48 hr ON INSOLUBLE NITROGEN CONTENT OF PUMPKIN MESOCARP

Cell fraction	Insoluble nitrogen (mg/10 g initial fresh wt.)		
		Disks aged for	
	Fresh disks	24 hr	48 hr
Extract	1·55*	2·24*	2·24*
Cell wall	1·02	1·27	1·66

* Prepared by precipitation with 5% trichloroacetic acid at 2°.

Incorporation of [^{14}C]-leucine into Protein

Disks, prepared and incubated aseptically, incorporated radioactivity from [^{14}C]-leucine into the cell-wall fraction and into the fraction of the extract that was insoluble in 5% trichloroacetic acid (Fig.1). The very high proportion of the added leucine that was incorporated is stressed, as is the fact that most of the incorporation took place in the first 24 hr of ageing. Evidence that this incorporation into both cell fractions represented protein synthesis is given below. First, treatment with either performic acid or 8 M urea did not reduce the radioactivity of either the cell-wall fraction or the precipitate obtained with trichloroacetic acid by more than 3 per cent of the initial values. Thus it is unlikely that the radioactivity was present as peptides bound to protein by disulphide linkages. Second, incubation in 5·8 N HCl at 100° followed by chromatography of the hydrolysate showed that complete hydrolysis of protein released all the radioactivity from the cell-wall fraction and the material precipitated by trichloroacetic acid as [^{14}C]-leucine. Third, partial hydrolysis of each cell fraction, followed by paper chromatography, gave chromatograms on which ninhydrin positive material appeared as a series of discrete bands spread between the origin and the solvent front. These bands almost certainly represented mixtures of peptides produced by the partial hydrolysis of protein. Examination of chromatograms of such partial hydrolysates of the cell-wall fraction and of the trichloroacetic acid precipitate showed that in both cases radioactivity was distributed throughout all the main bands of peptides. These experiments provide very strong

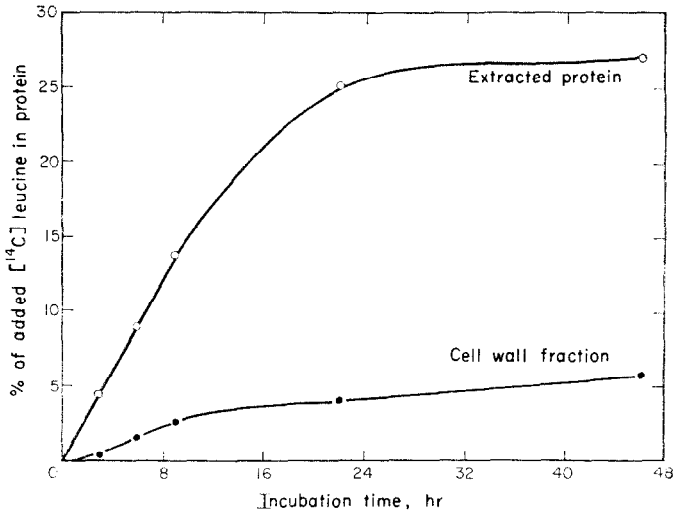

FIG. 1. INCORPORATION OF [2-^{14}C]-LEUCINE INTO PROTEIN BY DISKS OF PUMPKIN MESOCARP.

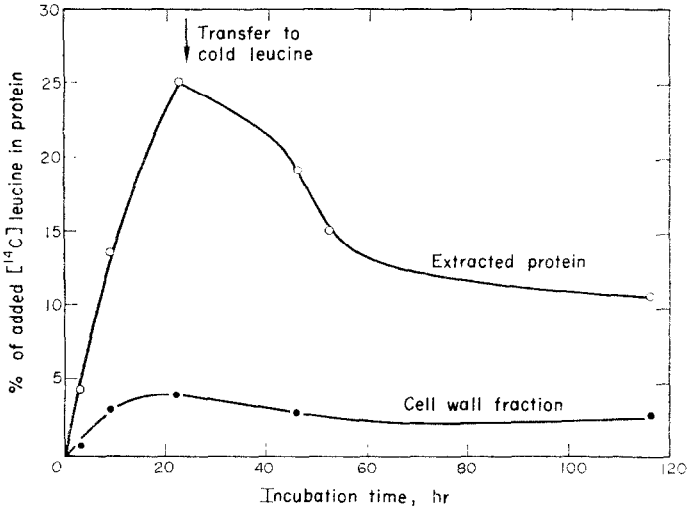

FIG. 2. EFFECT OF SUBSEQUENT INCUBATION IN UNLABELLED LEUCINE ON [2-^{14}C]-LEUCINE INCORPORATION INTO PROTEIN BY DISKS OF PUMPKIN MESOCARP.

evidence that the label in the cell-wall fraction and the trichloroacetic acid precipitate was present as [^{14}C]-leucine distributed throughout polypeptide chains.

The incorporation of leucine into protein during ageing could represent net protein synthesis, or a very high rate of turn-over of protein. Evidence against the latter possibility is provided by a pulse and chase experiment. Disks were incubated in [^{14}C]-leucine for the first 22 hr of ageing and were then transferred to non-radioactive leucine. The radioactivity found in protein at different stages of the pulse and chase is shown in Fig. 2. During the cold

chase the extractable protein lost some of its [^{14}C]-leucine but even after a cold chase of 100 hr 50 per cent of the activity originally incorporated was still present in protein. The incorporation into the cell-wall fraction was remarkably stable.

In view of the marked increase in protein that occurred on ageing, it was of interest to see whether this increase was preceded by an initial synthesis of the protein of any specific cellular organelle such as ribosomes. This question was investigated by following the incorporation of [^{14}C]-leucine into the protein of the main subcellular fractions during ageing. Samples of fifty disks that had been prepared and incubated aseptically in [^{14}C]-leucine were taken. Before extraction and fractionation these disks were thoroughly mixed with an excess of similar disks that had been incubated for the same time under non-sterile conditions in the absence of [^{14}C]-leucine. This procedure ensured that the leucine incorporation took place under aseptic conditions and yet gave sufficient material for reliable cell fractionation. The distribution of [^{14}C]-leucine in the protein of the main cellular fractions was very similar after 6, 24, and 48 hr of ageing (Table 4).

TABLE 4. INTRACELLULAR DISTRIBUTION OF [2-^{14}C]-LEUCINE INCORPORATED INTO PROTEIN BY DISKS OF PUMPKIN MESOCARP

Incubation time (hr)		^{14}C incorporated into protein in			
		2000 g ppt	20,000 g ppt	144,000 g ppt	144,000 g supernatant
6	Activity (cpm)	490	1220	1060	6140
	% Total incorporation into cell extract	5	14	12	69
24	Activity (cpm)	950	5400	3072	15300
	% Total incorporation into cell extract	4	22	12	62
48	Activity (cpm)	1180	8400	5700	23650
	% Total incorporation into cell extract	3	22	15	60

DISCUSSION

The difficulties involved in measuring the protein content of plant cells, mentioned in the introduction, are confirmed by my results. The incorporation of [^{14}C]-leucine into protein recovered in the cell-wall fraction shows that at least some of the nitrogen in this fraction was protein. The data do not allow us to decide whether this protein was a normal component of the cell wall or whether cytoplasmic protein became associated with the cell-wall fraction during extraction. None the less, the experimental data for pumpkin tissue are consistent with the view that ageing leads to the synthesis of long-lived protein that is very closely associated with the cell wall. Whatever may be the significance of the protein content of the cell-wall fraction, my results emphasize the need to analyse cell-wall fractions in any estimate of the total protein content of plant cells. Bell[6] found that different methods of separating protein and non-protein nitrogen in bran and flour gave different results. The data in Table 1 confirm that care is necessary in the selection of a technique for the isolation of protein from plants and cast doubt on experiments in which only one uncalibrated technique

[6] P. M. BELL, *Analyt. Biochem.* 5, 443 (1963).

has been employed. In particular the results weaken the evidence that the climacteric is accompanied by a net synthesis of total protein.[7–9] In the references quoted ethanol was used to separate protein from non-protein nitrogen. We cannot, now, exclude the possibility that the apparent net synthesis of protein was due to variation in the effectiveness with which the protein was separated from the non-protein nitrogen.

The evidence that the nitrogen in the dialysis residues, and in the precipitates obtained with trichloroacetic acid, was largely protein has been discussed. Although the nitrogen contents of the fractions obtained by these three methods do not agree exactly, there is very close agreement in that measurements by all three methods showed that ageing was accompanied by a marked increase in insoluble nitrogen. The results obtained by the Biuret method strongly indicate that this increase represented an increase in protein. The analysis of the cell-wall fractions shows that the increase in insoluble nitrogen in the extracts was not due to release of nitrogen from the cell-wall fraction. On the contrary, the data indicate that ageing caused a net synthesis of protein in both of the main cell fractions. This conclusion is fully supported by the demonstration that a very high proportion of exogenously supplied [^{14}C]-leucine was incorporated into protein in both fractions. The stability of the incorporation strongly indicates that it was not primarily due to protein turnover. The pattern of incorporation is that which would be expected if the increase in insoluble nitrogen represented net synthesis of protein.

Microbial contamination during ageing should be considered. In the experiments with [^{14}C]-leucine, no contaminants were detected even after 7 days' incubation on a general nutrient medium. This does not prove the absence of obligately endogenous micro-organisms or of contaminants that show no growth under the above conditions. However, the very high percentage of the added leucine that was incorporated into protein and the time course of the incorporation make it very unlikely that undetected micro-organisms contributed significantly to the results. Further, the close similarity between the increases in total protein and the rate of leucine incorporation, and the extent and time-course of the increase in total protein argue strongly against any marked contribution of micro-organisms to the measurements of total protein.

From the above results and discussion I conclude that slicing and incubation of the mesocarp of ripe pumpkin caused marked synthesis of protein that began within 3 hr of slicing and was largely complete within 24 hr. The distribution of [^{14}C]-leucine in the protein of the different cellular fractions indicates that the protein synthesis was not dependent upon an initial massive synthesis of either ribosomes or mitochondria. On the contrary the data suggest that ageing involved the synthesis of protein in all the main fractions of the cell.

It has been suggested that fruit ripening is due to the synthesis of specific proteins that catalyse the ripening process and the final degradation of the tissue.[10,11] The lack of information about ripening in pumpkin makes it difficult to relate my results to this process. The fact that disks of ripe pumpkin have been shown to be capable of considerable synthesis of protein is an unequivocal demonstration that senescent tissue of fruits can synthesize protein. As such this provides indirect evidence for the view that fruit ripening is an aspect of differentiation controlled by the ordered synthesis of specific proteins.

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Slices of pumpkin fruit are not unique in their ability to synthesize protein during ageing. Net synthesis of protein almost certainly occurs during the ageing of slices of potato tuber.[12,13] There is also evidence that net synthesis occurs in slices of a wide variety of other storage tissues.[14] The work of Hulme and his colleagues[15] strongly indicates that ageing of slices of apple peel results in protein synthesis. Extensive metabolic changes are known to accompany ageing of disks of both storage tissue[1,2] and apple peel.[15,16] In potato[13] and in apple peel,[15,16] many of the changes that occur during ageing are probably dependent upon the protein synthesis that occurs. Despite the general similarity of the ageing phenomena in all the tissues so far examined, it is clear that the specific events that occur in apple peel differ from those in the other tissues studied. In apple, ageing appears to cause the tissue to pass through a series of changes that are more closely related to the climacteric[11] than to the changes that occur during the ageing of storage tissue.[2] From the data presented in this and in a previous paper,[2] it seems that disks of pumpkin mesocarp resemble disks of storage tissue rather than disks of apple peel.

EXPERIMENTAL

Material

Ripe fruits of pumpkin (*Cucurbita pepo* L.) were bought from local stores and used at once. Disks (10 × 1 mm) of mesocarp were prepared by mechanical slicing of cylinders of tissue removed with a cork borer. Comparisons between fresh and aged tissue were made only within the same batch of replicate samples.

Ageing

For measurements of insoluble nitrogen, disks were aged by continuous circulation in 3 l. of aerated distilled water. The water was changed twice during the first 4 hr and at 12-hr intervals thereafter. For measurement of [^{14}C]-leucine incorporation, cores of mesocarp were removed aseptically as described by Gautheret[17] and then sliced under aseptic conditions in a glove-box. Preparation, manipulation, and incubation of all samples treated with [^{14}C]-leucine was carried out aseptically. Samples of twenty-one disks were incubated in 6·0 ml 0·18 mM DL-[2-^{14}C]-leucine (0·62 mc/mmole) dissolved in 0·033 M KH_2PO_4 at pH 5·2 and contained in 100-ml Erlenmeyer flasks stoppered with cotton wool plugs. The disks lay on filter paper supported by a single layer of glass beads (dia. 0·5 cm) on the bottom of the flask. The flasks were shaken reciprocally throughout the incubation at 60 cycles/min. In the pulse and chase experiment, samples of twenty-one disks incubated as described above were removed from the labelled leucine and transferred to flasks that contained 6·0 ml 10 mM DL-leucine in 0·033 M KH_2PO_4 at pH 5·2. This transfer and the incubation in the non-radioactive leucine were carried out aseptically. For the determination of the intracellular distribution of [^{14}C]-leucine incorporation, samples of fifty disks were prepared and incubated as described above except that the disks were placed in 250-ml Erlenmeyer flasks that contained 20 ml of the [^{14}C]-leucine solution. After preparing the samples of fifty disks aseptically, samples of 20-g fresh wt. were prepared and aged by circulation in aerated distilled water. The cores for these latter samples were taken from tissue immediately adjacent to that used for the preparation of the aseptic disks. All ageing was carried out at 26°.

All measurements of [^{14}C]-leucine incorporation are derived from samples of which representative disks showed no sign of contamination after 7 days' incubation at 27° on "Difco-Bacto" nutrient medium in 1% agar.

Measurement of Insoluble Nitrogen

Disks (160 g fresh wt.) were homogenized in 500 ml 0·066 M KH_2PO_4 (pH 7·0). The homogenate was filtered through a frittered glass filter and the cell debris re-extracted with two further 500-ml volumes of KH_2PO_4. The cell-wall fraction was taken for nitrogen assay without further treatment. The extracts were combined and divided into samples that were fractionated as follows: addition of 10% trichloroacetic acid at 2° followed by standing at 2° for 18 hr; addition of 5% trichloroacetic acid followed by heating at 98° for 20

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min; rapid addition to 3 volumes of boiling 98% ethanol, followed by boiling for 3 min; addition to 6 volumes of boiling water followed by boiling for 5 min; dialysis against 10 l. distilled water at 3° for 24 hr. Precipitates were collected by centrifugation and then taken for nitrogen assay. Nitrogen was estimated by a standard micro-Kjeldahl procedure using the digestion method of Hiller *et al.*[18] For determination of protein by the Biuret method[19] the precipitates were dissolved in 1·0 N NaOH. All values represent the mean of triplicate samples. All extracts were kept at 2° until the protein had been precipitated.

Incorporation of [^{14}C]*-Leucine*

At the end of the incubation the disks were rinsed with distilled water and then homogenized in 5·0 ml 0·066 M KH_2PO_4 (pH 7·0) that was 4 m-molar with respect to DL-leucine. The cell-wall fraction was removed by centrifugation and was re-extracted with two further 5·0 ml portions of KH_2PO_4. The extracts were combined and the protein was precipitated by the addition of an equal volume of 10% trichloroacetic acid at 2°. The protein precipitates and the cell-wall fraction were then washed thoroughly according to the procedure of Siekevitz[20] before assay of their radioactivity.

In measuring the distribution of [^{14}C]-leucine incorporation, samples of fifty disks were removed from [^{14}C]-leucine, rinsed, and then thoroughly mixed with 17-g fresh wt. of disks that had been incubated in the absence of [^{14}C]-leucine. The mixture was homogenized in 80 ml of a medium that contained at pH 8·1: sucrose (0·45 M), Tris (0·05 M), EDTA (0·01 M), and DL-leucine (4 mM). The homogenate was squeezed through two layers of muslin and fractionated by differential centrifugation. Each fraction was washed once and the washings were added to the supernatant before proceeding to the next stage of the fractionation. The protein in each fraction was precipitated and prepared for counting as described above.

In all experiments extraction and fractionation were carried out at 3°.

The characterization of the [^{14}C]-leucine incorporation was carried out with the protein precipitates, and the cell-wall fractions, after they had been washed by Siekevitz's procedure. For treatment with urea, samples equivalent to the protein precipitates and cell-wall fraction from twenty disks were suspended in 8 M urea at 20° for 3 hr. The urea was then removed by dialysis and the radioactivity of the dialysis residue was determined. Similar samples of extractable protein, and cell-wall fractions, were incubated for 50 min at 25° in a mixture of 5·0 ml 98% formic acid and 1·0 ml H_2O_2. The protein was recovered by precipitation with 5% trichloroacetic acid. For complete hydrolysis of protein, samples were refluxed in 5·8 N HCl for 24 hr. Partial hydrolysis was achieved by incubation in 11·6 N HCl at 37° for 52 hr. Chromatograms of the hydrolysates were run in butanol:acetic acid:water (4:1:1).

Measurement of Radioactivity

All measurements of ^{14}C were made after it had been converted to barium carbonate. The techniques used in the counting and in the conversion of carbon compounds to barium carbonate have been described.[21]

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