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NUCLEIC ACID SYNTHESIS AND INDUCED RESPIRATION BY DISKS OF CARROT STORAGE TISSUE

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Abstract—Net synthesis of RNA and DNA occurred when freshly prepared disks (1 × 10 mm) of storage tissue of carrot (*Daucus carota* L.) were incubated at 25° under moist conditions. Net synthesis of RNA occurred within 8 hr of preparation of the disks and continued for at least 4 days. Net synthesis of DNA was preceded by a lag of 48–72 hr. Incubation in either a simple combination of plant hormones, or in a nutrient medium known to support the growth of carrot tissue cultures, failed to overcome this lag. Incubation in 5-fluorouracil inhibited the net synthesis of RNA and DNA and the development of induced respiration. Incubation in cycloheximide inhibited the net synthesis of RNA and the development of induced respiration It is suggested that the development of induced respiration is dependent upon the synthesis of RNA and protein.

INTRODUCTION

A MARKED acceleration of metabolism occurs when thin slices of many plant tissues are incubated under moist conditions at physiological temperatures.[1,2] In this paper this phenomenon is called ageing. Although net synthesis of RNA has been reported to occur during ageing of carrot[3] and red beet[4] tissue, and there is evidence of a net synthesis of DNA during the ageing of disks of Jerusalem artichoke,[5] there is no detailed picture of the extent and timing of these syntheses. In particular we lack knowledge of DNA synthesis, and the relation between DNA and RNA synthesis in the same tissue. The work that is reported in this paper was undertaken in order to remedy this deficiency. An additional aim of the work was to investigate the relationship between any nucleic acid synthesis that occurred during ageing and the increased rate of respiration, induced respiration, that develops in the first 48 hr of ageing [6]

RESULTS

Effect of Ageing on Content of RNA and DNA

The amount of RNA in freshly cut disks varied widely. The RNA content was highest in young roots and fell during maturation and storage. The initial content of RNA of 14-week old carrots ranged from 600 to 800 μg/20 disks whereas the values for mature carrots, taken from storage clamps, ranged from 200 to 400 μg/20 disks. Regardless of the initial content of RNA, ageing was always accompanied by a marked net synthesis of

[1] G. G LATIES, in *Control Mechanisms in Respiration and Fermentation* (edited by B. WRIGHT), p 129, Ronald Press, New York (1963).
[2] T. AP REES, *Australian J. Biol Sci.* **19**, 981 (1966)
[3] C. J LEAVER and J. EDELMAN, *Biochem J* **97**, 27 p (1965).
[4] D. VAUGHAN and I R MACDONALD, *J Exptl Botany* **18**, 587 (1967).
[5] Y. MASUDA, *Plant Cell Physiol* **7**, 75 (1966)
[6] T. AP REES and H. BEEVERS, *Plant Physiol* **35**, 839 (1960)

TABLE 1 EFFECT OF AGEING ON NUCLEIC ACID CONTENT OF DISKS OF CARROT STORAGE TISSUE

hr aged	Number of experiments	RNA content as percentage that of fresh disks		DNA content as percentage that of fresh disks	
		Mean ± S E M.	Range	Mean ± S E.M	Range
8	1	112		92	
24	10	166 ± 11	103–209	95 ± 5	78–121
48	5	226 ± 28	137–302	98 ± 3	91–106
72	3	254 ± 11	235–272	159 ± 35	92–212
84	2	295	245–344	158	130–186
96	4	361 ± 33	302–426	162 ± 22	118–203

RNA. This net synthesis was evident within 8 hr of slicing and continued for at least 96 hr (Table 1). The extent and precise time course of synthesis varied with different batches of carrots. In general, net synthesis of RNA was most marked in tissue characterized by a low initial content of RNA (Fig. 1).

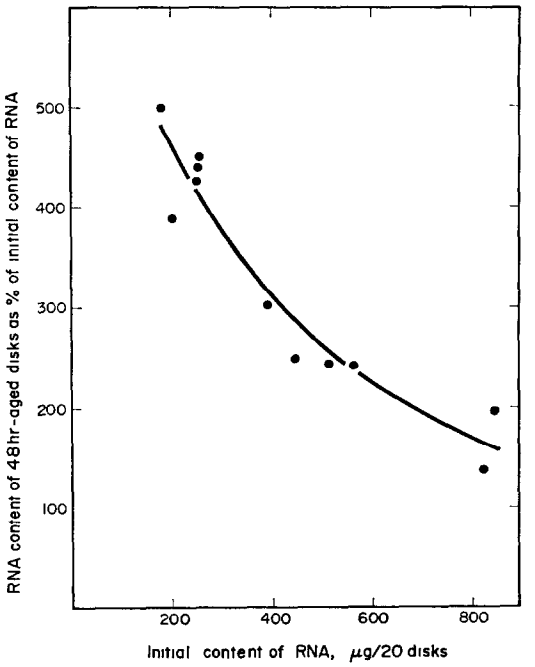

FIG 1 RELATIONSHIP BETWEEN RNA CONTENT OF FRESH DISKS AND THE EXTENT OF RNA SYNTHESIS IN 48 hr AGEING.

The amount of DNA in freshly cut disks also varied, but this variation was not obviously related to the age of the carrots. Ageing led to a net synthesis of DNA, the pattern of which differed from that of RNA synthesis (Table 1). First, net synthesis of DNA was less extensive than that of RNA. Second, net synthesis of DNA normally occurred only after a lag of 48–72 hr. We found no evidence of any relationship between the pattern of net synthesis of DNA and the amount of DNA in the freshly cut disks. We investigated whether the lag

phase preceding the onset of net synthesis of DNA could be overcome by ageing in the presence of hormones and nutrients. The amount of DNA in disks aged for 48 hr in 0·033 M KH_2PO_4 (pH 5·2) did not differ significantly from the amount in disks aged for the same time in the following media: kinetin (1·0 mg/l.); kinetin (0·2 mg/l.) and IAA (2·0 mg/l.); kinetin (1·0 mg/l.) and IAA (10 mg/l.); kinetin (0·2 mg/l.), IAA (2·0 mg/l.) and coconut milk (15%); Hildebrandt's[7] 'D' medium for tissue culture. The above patterns of net synthesis of RNA and DNA were found both in disks prepared and aged aseptically and in disks prepared and aged under non-sterile conditions.

Relations between Synthesis of Nucleic Acids and Protein, and Induced Respiration

The rapid rise in the content of RNA in the first 24 hr of ageing coincides with the development of induced respiration in carrot disks.[6] A further similarity between the development of induced respiration and the synthesis of RNA was discovered when we found that freshly cut disks of young carrots had a very high rate of respiration that increased only slightly during ageing (Table 2). As the carrot root matured the rate of respiration of freshly cut disks fell and the extent of the induced respiration rose. The possibility that the development of induced respiration was not only associated with synthesis of RNA, but was also dependent upon it, was tested by studying the effects of 5-fluorouracil on the two processes. 5-Fluorouracil effectively inhibited the development of induced respiration (Table 3) and the net synthesis of RNA and DNA (Table 4). Incubation of freshly cut disks and of 20 hr aged disks, in 5-fluorouracil (1 mg/ml) for 150 min had no detectable effect on the rates of oxygen uptake.

TABLE 2 RELATIONSHIP BETWEEN AGE OF CARROT AND THE DEVELOPMENT OF INDUCED RESPIRATION

Age of storage roots used for preparation of disks	Oxygen uptake (μl/hr/20 disks)	
	Freshly cut	Aged for 24 hr
Freshly harvested young roots, maximum diameter, 2·0 cm	246	267
Freshly harvested roots, maximum diameter, 4 0 cm	207	299
Mature roots from storage clamps	100	286

We have shown that ageing of carrot disks is accompanied by protein synthesis.[8] This fact, together with the above demonstration of a relationship between induced respiration and the synthesis of RNA, prompted us to investigate whether the development of induced respiration and the synthesis of RNA were dependent upon the protein synthesis. We did this by measuring the effect of cycloheximide, known to inhibit the protein synthesis,[8] on the two processes. The data in Tables 3 and 4 show that cycloheximide inhibited the development of induced respiration and the synthesis of RNA. The effects on respiration were reversible in that removal of the cycloheximide was followed by the development of an increased rate of respiration.

[7] A. C. HILDEBRANDT, A. J RIKER and B M. DUGGAR, *Am J Botany* **33**, 591 (1946).
[8] T AP REES and J A BRYANT, *Phytochem* **10**, 1183 (1971)

TABLE 3. EFFECTS OF 5-FLUOROURACIL AND CYCLOHEXIMIDE ON DEVELOPMENT OF INDUCED RESPIRATION BY DISKS OF CARROT STORAGE TISSUE

Oxygen (μl) absorbed per hr by 20 fresh disks	Incubation media used during ageing	Oxygen (μl) absorbed per hr by 20 disks aged for. 21 hr	27 hr	45 hr
110	0 02 M KH_2PO_4 (pH 5·2)	168	153	182
	5-Fluorouracil (1 mg/ml) in 0 02 M KH_2PO_4 (pH 5 2) for 21 hr followed by 24 hr in 0 02 M KH_2PO_4(pH 5·2)	112	113	93
108	0·02 M KH_2PO_4 (pH 5·2)	210	196	—
	Cycloheximide (10 μg/ml) in 0·02 M KH_2PO_4 (pH 5·2) for 21 hr followed by 6 hr in 0·02 M KH_2PO_4 (pH 5·2)	150	208	—

TABLE 4. EFFECTS OF 5-FLUOROURACIL AND CYCLOHEXIMIDE ON NUCLEIC ACID SYNTHESIS DURING AGEING OF DISKS OF CARROT STORAGE TISSUE

Nucleic acid content (μg) of 20 fresh disks		Incubation media used during ageing	Nucleic acid content (μg) of 20 disks aged for 48 hr		96 hr	
RNA	DNA		RNA	DNA	RNA	DNA
447	98	0 02 M KH_2PO_4 (pH 5 2)	1120	90	1746	199
		5-Fluorouracil (1 mg/ml) in 0·02 M KH_2PO_4 (pH 5 2)	748	98	840	97
390	—	0 02 M KH_2PO_4 (pH 5·2)	1185	—	—	—
		Cycloheximide (10 μg/ml) in 0 02 M KH_2PO_4 (pH 5 2)	634	—	—	—

DISCUSSION

Our results establish that ageing of carrot storage tissue is accompanied by a massive net synthesis of RNA. The continued net synthesis of RNA after more than 3 days ageing distinguishes our results from those reported for carrot tissue by Leaver and Edelman[3] and for red beet tissue by Vaughan and MacDonald.[4] The reason for these differences is not apparent but we stress the frequency with which we found net synthesis of RNA continuing after more than 3 days ageing. The results obtained with cycloheximide indicate that the net synthesis of RNA is at least partially dependent upon the synthesis of protein. The work of Leaver and Key,[9] the evidence that 5-fluorouracil inhibits the formation of ribosomal RNA,[10] and the general consideration that the bulk of cellular RNA is normally ribosomal RNA, lead us to suggest that much of the RNA formed during ageing in carrot disks represents the synthesis of ribosomes

Our results also show that ageing of carrot disks leads to a net synthesis of DNA and that the time course of this net synthesis differs radically from that of RNA synthesis. Our

[9] C J LEAVER and J. L. KEY, *Proc Natl Acad Sci.* **57**, 1338 (1967).
[10] J L. KEY and J. INGLE, *Proc. Natl Acad Sci.* **52**, 1382 (1964).

failure to overcome the lag in net synthesis of DNA, not only with simple combinations of hormones but also with a nutrient medium known to support the proliferation of carrot root cells,[11] suggests that considerable changes are necessary before dormant carrot storage tissue can synthesize DNA. The inhibition of DNA synthesis by 5-fluorouracil may mean that RNA synthesis is required before DNA can be made. Alternatively it may reflect a direct effect of 5-flourouracil on DNA synthesis caused by the inhibition of thymidylate synthetase by 5-fluoro-2'-deoxyuridine 5'-monophosphate.[12] The separation, by at least 2 days, of the onset of net synthesis of DNA from that of RNA indicates that the mechanisms that control the synthesis of the two nucleic acids are independent, and suggests that aged disks of carrot may be suitable material for the study of such mechanisms.

We have shown that 5-fluorouracil effectively inhibits the synthesis of RNA during ageing and that it has no direct effect upon the rate of respiration of either fresh or aged disks. Consequently we think that the ability of 5-fluorouracil to inhibit the development of induced respiration is strong evidence that the increase in the rate of respiration is at least partially dependent upon the synthesis of RNA. We have already demonstrated that protein synthesis occurs during ageing of carrot disks and that the preparation of cycloheximide used in the experiments reported in this paper inhibited this synthesis.[8] In addition we have produced very strong evidence that this inhibition is due to a specific effect of the cycloheximide on the polymerization of amino acids and not to any direct effect of cycloheximide on respiration. The reversibility of the effect of cycloheximide on the development of induced respiration (Table 3) is evidence that this effect is not due to any general and permanent damage to metabolism. We think that our results with cycloheximide provide sound evidence that the development of induced respiration in carrot disks is, to a significant extent, dependent upon the synthesis of protein as well as the synthesis of RNA. Thus in both carrot and potato[13] tissue there is very strong evidence that the development of induced respiration requires the synthesis of protein and RNA. Laties[1] has suggested that induced respiration is due to the escape of some volatile inhibitor of respiration during ageing. The results obtained with carrot and potato make it clear that such an explanation, on its own, is inadequate. If the removal of an inhibitor of respiration were all that were necessary for the development of induced respiration then it is very unlikely that the development would be sensitive to inhibitors of protein and RNA synthesis.

Our results show that the onset of dormancy in carrot roots is accompanied by a marked decline in both RNA content and the rate of respiration. In general terms the ageing phenomenon may be viewed as a reversal of the changes that accompany dormancy. It seems likely that ageing represents a change from dormancy to rapid growth that is eventually checked by lack of nutrients or hormones.

EXPERIMENTAL

Material

Carrots (*Daucus carota* L) were bought locally and were used at once Cylinders of storage tissue were taken vertically and sliced to give disks 1 × 10 mm Disks were aged at 25° by incubation in 7 0 ml KH_2PO_4 (0 033 M or 0 02 M) at pH 5·2 in 100 ml Erlenmeyer flasks stoppered with cotton wool plugs Samples of twenty disks were laid on filter paper supported by a single layer of glass beads (diameter 0 4 cm) The flasks were shaken reciprocally throughout the incubation Inhibitors and hormones were dissolved in KH_2PO_4 (0 033 M or 0 02 M) and the pH was adjusted to pH 5 2. The methods used in the preparation and incubation

[11] D K Dougall, *Exptl. Cell Res* **33**, 438 (1964).
[12] C Heidelberger, in *Chemotherapy of Cancer* (edited by P A. Plattner), p 88, Elsevier, Amsterdam (1964)
[13] R. E Click and D P. Hackett, *Proc Natl Acad Sci* **50**, 243 (1963)

of disks under aseptic conditions, and in the detection of contaminants, have been described.[14] Comparisons between fresh and aged tissue were made only within the same batch of replicate samples.

Extraction and Assay of Nucleic Acids

We compared a number of methods for extraction of nucleic acids and found that a modification of Cherry's[15] method was the most satisfactory for this tissue The methods of Schmidt and Thannhauser[16] and of Ogur and Rosen[17] gave poor separation of RNA from DNA and yielded extracts which, judged by their u.v. absorption spectra, were heavily contaminated with u v absorbing impurities Extracts prepared by the method of Fowler and ap Rees[18] failed to show absorption maxima in the region 255 to 260 nm; the yield of DNA was very low. A method which combined features of Kirby's[19] technique and Guinn's[20] technique gave a low yield of both nucleic acids

The extraction procedure started with the killing and homogenisation of samples of twenty disks in methanol The initial temperature of the methanol was −20° but rose to 20° during the homogenisation. The disks were homogenised by grinding first in a pestle and mortar and then in a hand-model glass Ten-Broek homogeniser The homogenate was extracted successively with methanol at 20° (three times), 0·31 N perchloric acid at 2°, ethanol at −20°, ethanol–ether mixture (2 1) at 50° for 30 min, and ethanol at 20° Next the nucleic acids were removed from the homogenate by extraction with 0·77 N perchloric acid (two 15-min extractions at 90° followed by one 2-min extraction at 20°). The total amount of nucleic acid in the extract was deter-

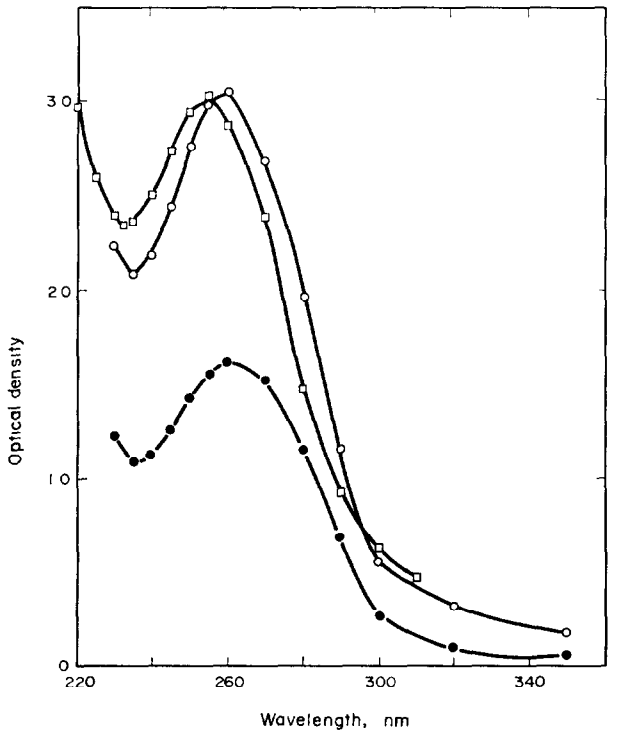

FIG 2. U V. ABSORPTION SPECTRA OF NUCLEIC ACID EXTRACTS OF FRESH AND AGED DISKS OF CARROT. The nucleic acids were extracted from samples of 20 disks and the extracts were made up to 15 ml. ● Fresh disks, ○ 47-hr aged disks, □ 95-hr aged disks.

[14] T AP REES, *Phytochem* **8**, 1879 (1969).
[15] J H CHERRY, *Plant Physiol* **37**, 670 (1962)
[16] G SCHMIDT and S J. THANNHAUSER, *J Biol Chem* **161**, 83 (1945)
[17] M OGUR and G ROSEN, *Archs Biochem* **25**, 262 (1950).
[18] M W FOWLER and T. AP REES, *Biochem Biophys Acta* **201**, 33 (1970)
[19] K S KIRBY, *Prog Nucleic Acid Res.* **3**, 1 (1964)
[20] G GUINN, *Plant Physiol* **41**, 689 (1966).

mined from measurements of absorbance at 260 nm. DNA was assayed by the diphenylamine reaction [21] RNA was determined by subtracting the values for DNA from those for total nucleic acid. All values for DNA and RNA represent means from three or two replicate samples

The methods that we used to assay nucleic acids are susceptible to interference from impurities in the extracts Sugars and uronic acids could interfere with the estimation of DNA by the diphenylamine method. Paper chromatography of our nucleic acid extracts in EtOAc–pyridine–H_2O (8 2 1) revealed only minute traces (< 10 μg/ml) of sugars and uronic acids Less than 2 per cent change in absorbance at 600 nm was caused by 2 mg of arabinose [21] We found that as much as 1 mg/ml of glucuronic acid was necessary to alter the absorbance at 600 nm by 21 per cent. Thus we argue that our assays of DNA were not significantly affected by contaminating sugars or uronic acids Our estimates of total nucleic acid would be affected by the presence of substances that absorbed at 260 nm The absorption spectra of extracts from fresh and aged disks compared favourably with spectra of RNA that had been purified from yeast (Sigma Chemical Co and Koch-Light Laboratories Ltd) and hydrolysed in 0 77 N perchloric acid (Fig 2) These spectra strongly indicate that our extracts did not contain impurities that contributed appreciably to absorbance at 260 nm Since the most likely source of interference was protein[22] we determined the protein present in the extracts[23] and, using the extinction coefficient at 260 nm of purified bovine serum albumin, estimated the contributions that protein in the nucleic acid extracts made to absorbance at 260 nm On this basis we found that the percentage of absorbance at 260 nm that could be attributed to protein was 6 5, 8 1 and 6 8 per cent in extracts of fresh, 47-hr aged, and 95-hr aged disks, respectively We consider these contributions to be very small in relation to the changes in nucleic acid content that we found.

Measurement of Oxygen Uptake

Oxygen uptake was determined manometrically at 25° by Warburg's direct method All values represent means of rates, measured over at least 1 hr, of triplicate samples

Acknowledgement—J. A B. thanks the Science Research Council for financial support

[21] K Burton, *Biochem. J.* **62**, 315 (1956).
[22] H N Munro and A Fleck, in *Methods of Biochemical Analysis* (edited by D Glick), Vol. XIV, p 113, Interscience, New York (1966).
[23] E Layne, in *Methods in Enzymology* (edited by S. P. Colowick and N O Kaplan), Vol III, p 447, Academic Press, New York (1957).