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EFFECTS OF CYCLOHEXIMIDE ON PROTEIN SYNTHESIS AND RESPIRATION IN DISKS OF CARROT STORAGE TISSUE

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Abstract—This paper describes experiments that were undertaken in order to discover whether, in disks (1 × 10 mm) of storage tissue of carrot (*Daucus carota* L), cycloheximide inhibited protein synthesis directly, or indirectly via an effect on respiration. Freshly cut disks, incubated in water for 4 hr and then transferred for 75 min to a medium that contained [^{14}C]-leucine, incorporated [^{14}C]-leucine into protein. Cycloheximide (10 μg/ml) caused a 95 per cent inhibition of this incorporation In comparable experiments, the effects of cycloheximide on the distribution of ^{14}C from [6-^{14}C]-glucose were determined. The results indicate very strongly that cycloheximide inhibited protein synthesis directly and not via any effect on respiration. Cycloheximide did not increase the rate of oxygen uptake It is concluded that protein synthesis occurs when freshly cut disks of carrot are incubated in water and that cycloheximide inhibits this synthesis directly.

INTRODUCTION

CYCLOHEXIMIDE inhibits protein synthesis in a variety of eucaryotic cells[1] and has been shown to interfere with the transfer of amino acids from aminoacyl-tRNA to nascent polypeptide chains [2] There is evidence that sensitivity to cycloheximide is a property of the 60 s ribosomal sub-unit.[3] In experiments with higher plants, sensitivity of a particular activity to cycloheximide has frequently been used as evidence that this activity depends directly on protein synthesis.[4] This argument rests, to a considerable extent, on the extrapolation to higher plants of conclusions based on experiments with micro-organisms and animals. Recently, MacDonald and Ellis[4] have challenged the validity of this extrapolation by pointing out that there is no proof that cycloheximide specifically inhibits protein synthesis in higher plants in general, and by providing evidence that cycloheximide may inhibit protein synthesis indirectly by interfering with respiration in certain plants.

Extensive changes occur when thin slices of many plant tissues are incubated under moist conditions.[5] We refer to this phenomenon as ageing. We have been using cycloheximide in attempts to discover whether any of the events that occur during ageing of disks of carrot storage tissue are dependent upon protein synthesis. This is one of the tissues in respect of which MacDonald and Ellis suggest that cycloheximide may inhibit protein synthesis indirectly via an effect on respiration.[4] Consequently we have investigated the

[1] H. D. SISLER and M. R. SIEGEL, in *Antibiotics* (edited by D. GOTTLIEB and P. D SHAW), Vol. 1, p 283, Springer-Verlag, Berlin (1967).
[2] W MCKEEHAN and B. HARDESTY, *Biochem. Biophys Res. Commun.* **36**, 625 (1969).
[3] S S. RAO and A. P. GROLLMAN, *Biochem. Biophys Res. Commun.* **29**, 696 (1967)
[4] I. R. MACDONALD and R. J ELLIS, *Nature* **222**, 791 (1969)
[5] T. AP REES, *Australian J Biol Sci.* **19**, 981 (1966)

effects of cycloheximide on protein synthesis and respiration during the ageing of carrot disks. There were two aims to the work. The first was to establish whether cycloheximide inhibited protein synthesis during ageing. This question was examined by measuring the effect of cycloheximide on the incorporation of [^{14}C]-leucine into protein. The second aim was to see whether any such inhibition of protein synthesis was due to interference with energy transfer in respiration rather than to an effect of cycloheximide on the mechanism of protein synthesis. This question was investigated by determining the effects of cycloheximide on oxygen uptake and on the distribution of label from [6-^{14}C]-glucose. If cycloheximide acted as an uncoupling agent towards carrot tissue then cycloheximide and 2,4-dinitrophenol would probably affect oxygen uptake in the same way. In addition we think that if cycloheximide inhibited protein synthesis primarily via an effect on respiration, then this would be reflected in inhibition of the movement of label from [6-^{14}C]-glucose into all compounds that require respiratory energy for their synthesis. Thus, we paid particular attention to the movement of label into sucrose as a criterion of whether cycloheximide affected energy transfer during respiration.

RESULTS

Effects on Protein Synthesis

Duplicate samples of disks were incubated in water for 220 min. Then one sample was suspended in 0·02 M KH_2PO_4 at pH 5·2 and the other in a solution of cycloheximide (10 μg/ml) in 0·02 M KH_2PO_4 at pH 5·2. After 65 min of this pretreatment the phosphate and the cycloheximide solutions were replaced, respectively, with solutions of 0·2 mM L-[U-^{14}C]-leucine in 0·02 M KH_2PO_4 at pH 5·2, and 0·2 mM L-[U-^{14}C]-leucine in 0·02 M KH_2PO_4 at pH 5·2 that contained cycloheximide at 10 μg/ml. Uptake and incorporation into protein were determined after a 75 min incubation in the [^{14}C]-leucine (Table 1). An appreciable proportion of the absorbed leucine was incorporated into the protein fraction. We present the following evidence that this incorporation represented protein synthesis. Treatment with 8 M urea did not reduce the radioactivity of the protein fraction. Thus it is unlikely that the radioactivity was present as peptides bound to the protein by disulphide linkages. Complete hydrolysis of the protein fraction, followed by paper chromatography of the hydrolysate, showed that all the radioactivity in the protein fraction was recovered from the area of the chromatogram that coincided with leucine. Partial hydrolysis of the

TABLE 1. EFFECT OF CYCLOHEXIMIDE ON INCORPORATION OF [^{14}C]-LEUCINE INTO PROTEIN BY DISKS OF CARROT STORAGE TISSUE

	Radioactivity (counts/min/21 disks)	
	Control	Cycloheximide
Activity supplied	449,960	449,960
Activity recovered in		
(i) Medium at end of incubation	184,425	340,075
(ii) Protein	25,280	524
Uptake	265,535	109,885
Activity in protein as percentage of uptake	9·5	0 48

protein fraction, followed by paper chromatography of the hydrolysate, gave chromatograms on which ninhydrin-positive material appeared as a series of bands spread between the origin and the solvent front. These bands are taken to represent mixtures of peptides produced by the partial hydrolysis. The radioactivity originally present in the protein fraction was distributed throughout all the bands of peptides. From these results we argue that the radioactivity in the protein fraction was present as [^{14}C]-leucine distributed throughout polypeptide chains. Incubation in cycloheximide significantly reduced the uptake of [^{14}C]-leucine. Even when this is taken into account, the results show that cycloheximide caused a 95 per cent inhibition of the incorporation of [^{14}C]-leucine into protein.

Effects on Metabolism of [6-^{14}C]-*Glucose*

Samples were prepared from the same batch of carrots that was used in the experiments with [^{14}C]-leucine. These samples were aged in water and pre-treated with cycloheximide in exactly the same way as in the [^{14}C]-leucine experiments. At the end of the pre-treatment one sample was suspended in 0·1 mM [6-^{14}C]-glucose in 0·02 M KH_2PO_4 (pH 5·2) and the other sample was suspended in 0·1 mM [6-^{14}C]-glucose in 0·02 M KH_2PO_4 (pH 5·2) that contained cycloheximide at 10 μg/ml. The general distribution of label at the end of a 75 min incubation is shown in Table 2. We accounted for essentially all of the ^{14}C supplied to each sample. Thus the labelling patterns in Table 2 are not due to losses during analysis.

TABLE 2 EFFECT OF CYCLOHEXIMIDE ON DISTRIBUTION OF LABEL FROM [6-^{14}C]-GLUCOSE SUPPLIED TO DISKS OF CARROT STORAGE TISSUE

	Control		Cycloheximide	
Fraction	^{14}C (counts/min/ 21 disks)	^{14}C as % [^{14}C]-glucose metabolized	^{14}C (counts/min/ 21 disks)	^{14}C as % [^{14}C]-glucose metabolized
Activity supplied	380,240		380,240	
[^{14}C]-Glucose metabolized*	139,200		54,380	
Activity recovered in:				
(i) CO_2	4700	3	1400	3
(ii) Ethanol-insoluble fraction	35,100	25	6950	13
(iii) Water-soluble fraction				
Basic components	23,350	17	6790	12
Acidic components	35,500	26	10,640	20
Neutral components	40,550	29	28,600	52
(iv) Medium at end of incubation	246,050		321,000	
Total activity recovered	385,250		375,380	

* This figure represents the sum of the ^{14}C recovered in CO_2, the ethanol-soluble fraction and the ethanol insoluble fraction that were prepared as described in Experimental.

Incubation in cycloheximide decreased the uptake of glucose and had the following effects on the distribution of the label that was absorbed. Firstly, incorporation into the fraction insoluble in 80 per cent ethanol was halved. Secondly, incorporation into the basic and acidic components of the water soluble fraction was reduced by about 25 per cent. Thirdly, incorporation into the neutral fraction was substantially increased. Paper chromatography

TABLE 3. EFFECT OF CYCLOHEXIMIDE ON DISTRIBUTION OF LABEL IN NEUTRAL FRACTION AFTER SUPPLYING [6-^{14}C]-GLUCOSE TO DISKS OF CARROT STORAGE TISSUE

Fraction	Control		Cycloheximide	
	^{14}C, cpm	^{14}C as % ^{14}C added to chromatogram	^{14}C, cpm	^{14}C as % ^{14}C added to chromatogram
^{14}C added to chromatogram	1095		595	
^{14}C recovered in:				
Sucrose	793	72	370	62
Glucose	157	14	150	25
Fructose	87	8	33	5
Remainder of chromatogram	26	2	20	3
Total ^{14}C recovered from chromatogram	1063	97	573	96

of portions of the neutral fractions revealed the detailed distribution of ^{14}C in these fractions (Table 3). Recovery of the radioactivity added to the chromatograms was almost complete. This fact, and the complete recoveries shown in Table 2, mean that the data in Tables 2 and 3 may be used to show that incubation in cycloheximide increased the percentage of the metabolized ^{14}C that was found in sucrose from 21 to 32 per cent.

Effects on Oxygen Uptake

We measured the effect of cycloheximide on the oxygen uptake of fresh, 24-hr-aged, and 96-hr-aged disks (Table 4). Cycloheximide at either 1 μg/ml, the concentration used by MacDonald and Ellis,[4] or at 10 μg/ml, the concentration used in our experiments, did not increase the rate of oxygen uptake within 120 min. The addition of 2,4-dinitrophenol to identical samples of disks led to immediate increases in oxygen uptake. The only short term effect of cycloheximide on oxygen uptake that we detected was the inhibition of the

TABLE 4 EFFECTS OF CYCLOHEXIMIDE AND 2,4-DINITROPHENOL ON OXYGEN UPTAKE OF DISKS OF CARROT STORAGE TISSUE

Treatment of disks	min after addition of inhibitor	Oxygen uptake (μl/hr/25 disks)				
		Control	Cycloheximide (1 μg/ml)	Cycloheximide (10 μg/ml)	Dinitrophenol (4 6 μg/ml)	Dinitrophenol (4 6 μg/ml) and cycloheximide (10 μg/ml)
Freshly cut	30–90	140	121	119	214	—
	90–150	132	121	123	208	—
	150–210	163	113	120	219	—
	210–270	175	111	124	212	—
Aged for 24 hr	10–70	244	255	252	300	—
	70–130	241	250	256	284	—
Aged for 96 hr	0–60	236	241	244	—	267
	60–120	232	235	242	—	—

start of induced respiration in freshly cut disks.[5] In their experiments,[4] MacDonald and Ellis (personal communication) aged carrot disks in water and measured respiration rates of disks that were in water or in aqueous solutions of cycloheximide. In our experiments the disks were suspended in 0·02 M KH_2PO_4. Thus our failure to observe a stimulation of oxygen uptake by cycloheximide might have been due to a stimulatory effect of phosphate on the respiration of both the control and the cycloheximide treated samples. In experiments with freshly cut disks of carrot and with disks that had been aged aseptically in water for 24 hr, we found that the addition of 0·02 M KH_2PO_4 (pH 5·2) to disks suspended in water did not alter the rate of oxygen uptake in the 2 hr following the addition of phosphate. Thus the fact that cycloheximide did not stimulate oxygen uptake in our experiments cannot be attributed to a stimulation of respiration by the phosphate solutions in which we suspended the disks.

DISCUSSION

Our experiments with [^{14}C]-leucine establish that protein synthesis occurs during the early stages of ageing in carrot storage tissue. These results and those of MacDonald, Knight and DeKock,[6] show that thin disks of carrot storage tissue resemble those of pumpkin mesocarp[7] and potato tuber[8] in that incubation at physiological temperatures rapidly leads to a marked synthesis of protein. The fact that this phenomenon has now been demonstrated in three such different tissues indicates that it may be widespread and must be taken into account in interpreting results obtained with slices or sections of plant tissues in general.

Although cycloheximide inhibited the uptake of leucine, it had a much greater inhibitory effect on the incorporation into protein of the leucine that was absorbed by the tissue. We conclude from these experiments that cycloheximide caused a very rapid and almost complete inhibition of protein synthesis. There is also evidence that cycloheximide inhibits protein synthesis in other higher plants.[9–11]

A demonstration that cycloheximide inhibits protein synthesis *in vivo*, or even *in vitro*, does not answer the charge that the inhibition *in vivo* may be due to interference with respiration. We think that this question can be resolved by experiments *in vivo* that provide evidence that cycloheximide does not interfere with respiration under conditions in which it inhibits protein synthesis. There are three reasons why we believe that our inhibition of protein synthesis by cycloheximide was not primarily due to interference with respiration. Firstly, cycloheximide, in contrast to dinitrophenol, did not increase the rate of oxygen uptake. If cycloheximide had acted primarily as an uncoupling agent then it is probable that this would have been revealed by an increase in oxygen uptake similar to that produced by dinitrophenol. Secondly, cycloheximide not only failed to inhibit the accumulation in sucrose of label from metabolised [^{14}C]-glucose, but actually increased it by as much as 50 per cent. If cycloheximide had impaired energy transfer so that this caused almost complete inhibition of protein synthesis, then it is extremely likely that other endergonic processes, such as sucrose synthesis, would have been similarly inhibited. Finally, the effects that cycloheximide did have on the metabolism of [6-^{14}C]-glucose are those that would be expected to

[6] I. R MacDonald, A H. Knight and P C. DeKock, *Physiol. Plantarum* **14**, 7 (1961).
[7] T ap Rees, *Phytochem.* **8**, 1879 (1969)
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[9] J L Key, N. M Barnett and C Y Lin, *Ann N Y Acad Sci* **144**, 49 (1967).
[10] G M Polya, *Australian J. Biol Sci.* **21**, 1107 (1968).
[11] M Zucker, *Plant Physiol* **44**, 912 (1969).

follow from an inhibition of protein synthesis. The increased contribution to sucrose may be ascribed partly to an inhibition of the development of induced respiration,[12] and partly to an inhibition of the development of acid invertase activity.[13] The decreased incorporation into the alcohol-insoluble fraction would result from a direct inhibition of protein synthesis. The incorporation into this fraction that took place in the presence of cycloheximide probably represents the formation of insoluble carbohydrates.[14] The small decreases in incorporation into the acidic and basic fractions may be attributed to diversion of more of the absorbed glucose into sucrose rather than into respiratory pathways. In addition, feedback inhibition of amino acid synthesis may have been caused by the severe inhibition of protein synthesis.

We do not think that the inhibition of the uptake of leucine and glucose by cycloheximide shows that cycloheximide interfered with respiration. The ability of disks of storage tissue to absorb at least some solutes increases dramatically during ageing.[15,16] Cycloheximide inhibits many of the events that occur during ageing (J. A. Bryant and T. ap Rees, unpublished observations). Thus the inhibition of the uptake of glucose and leucine may stem from an inhibition of the development of an increased ability to absorb solutes. In addition it is conceivable that inhibition of protein synthesis could inhibit solute uptake either because the mechanism of uptake involves protein synthesis or because uptake depends upon the activity of protein that turns over rapidly.[17]

We conclude that the cycloheximide that we have used in our experiments with carrot disks inhibited protein synthesis and that it is improbable that this inhibition was due primarily to effects on respiration. We do not think that the data of MacDonald and Ellis[4] demonstrate a direct effect of cycloheximide on respiration. We argue that the present limited knowledge of the control and interaction of protein synthesis, respiration, and solute uptake is inadequate for us to decide whether changes in the uptake of oxygen or of salts are directly related to energy transfer or to protein synthesis. The difficulties involved in interpreting an inhibition of solute uptake have already been discussed. The respiratory data are also open to alternative explanation. For example, MacDonald and Ellis[4] found appreciable stimulation of respiration only in disks of storage tissue that had been aged. Such tissue may have been, to some extent, *in extremis*. It is possible that almost complete inhibition of protein synthesis in such tissue could reduce, critically, the content of some rapidly turning-over protein necessary for maintaining the coupling between ATP synthesis and electron transport. Alternatively, inhibition of protein synthesis could increase respiration by making available more respiratory substrate. These explanations receive some support from the speed with which cycloheximide inhibited protein synthesis in our experiments and from the fact that MacDonald and Ellis[4] found a 30-min lag between the addition of cycloheximide and the increase in respiration

The effects of puromycin on plant tissues should be considered in relation to our arguments. As an inhibitor of protein synthesis in disks of red beet, puromycin is contrasted favourably with cycloheximide by MacDonald and Ellis [4] We think that this is a misleading comparison. The mode of action of puromycin appears to differ radically from that of

[12] T. AP REES and H. BEEVERS, *Plant Physiol.* **35**, 839 (1960).
[13] C P P RICARDO and T AP REES, *Phytochem* **9**, 239 (1970)
[14] T AP REES and H BEEVERS, *Plant Physiol* **35**, 830 (1960).
[15] B. C. LOUGHMAN, *Plant Physiol* **35**, 418 (1960)
[16] B. R GRANT and H. BEEVERS, *Plant Physiol.* **39**, 78 (1964).
[17] E A. C. MACROBBIE, *Australian J Biol Sci* **19**, 371 (1966).

cycloheximide.[18] In addition, the concentration of puromycin, shown to have little effect on chloride uptake by beet disks, appears to have caused only a 30% inhibition of the increase in insoluble nitrogen that occurred during ageing.[19] Thus the failure of puromycin to produce the effects reported for cycloheximide may have been due to a failure of puromycin to inhibit protein synthesis as rapidly and extensively as did cycloheximide.

EXPERIMENTAL

Material

Cycloheximide was obtained from Koch-Light Laboratories, Colnbrook, Bucks. D-[6-^{14}C]-Glucose and L-[U-^{14}C]-leucine were obtained from the Radiochemical Centre, Amersham, Bucks

Carrots (*Daucus carota* L.) were bought locally For measurements of respiration, cylinders of storage tissue were taken vertically and sliced to give disks (10 × 1 mm) that were aged by gentle circulation in aerated distilled water at 25°. For the experiments with labelled substrates similar disks were prepared under aseptic conditions as described earlier [7] The disks were maintained under aseptic conditions until the end of the incubation in the labelled substrates All data in this paper that relate to the metabolism of labelled substrates are derived from samples of which representative disks, removed at the end of the incubation in the labelled substrates, showed no sign of contamination after 18 days incubation at 25° on 'Difco-Bacto' nutrient medium in 1% agar

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

Duplicate samples of 21 disks were suspended in 7 0 ml of medium in 100-ml Erlenmeyer flasks that were stoppered with cotton wool plugs and shaken gently at 25°. The experimental treatments are described in Results. The specific activity of the [^{14}C]-leucine was 5 0 mc/mmole. After incubation in [^{14}C]-leucine, the medium was removed and the samples were rinsed quickly with 0 02M KH_2PO_4 (pH 5 2). The washings were added to the medium and uptake of [^{14}C]-leucine was determined by measuring the difference between the radioactivity of this solution and the radioactivity added at the beginning of the incubation After removal of the washing solution the disks were added to 50 ml 5 per cent trichloroacetic acid (2°) that was 5 mM with respect to L-leucine, and left at 4° for 24 hr. The disks were then homogenized in 5 per cent trichloroacetic acid and the insoluble material was washed thoroughly according to the procedure of Siekevitz[20] before assay of its radioactivity The methods used to characterize the incorporation of [^{14}C]-leucine into this insoluble material have been described [7]

Metabolism of [6-^{14}C]-*Glucose*

Replicate samples of 21 disks were set up as in the experiments with [^{14}C]-leucine and were treated as described in Results The specific activity of the [6-^{14}C]-glucose was 14 mc/mmole $^{14}CO_2$ was collected in KOH in centre wells. At the end of the incubation the [6-^{14}C]-glucose solutions were removed and the disks were rinsed with 0 02 M KH_2PO_4 (pH 5·2). The washings were added to the medium and the resulting solutions are described as 'medium at the end of the incubation' After removal of the washing solutions the disks were killed and extracted for 2 min with boiling 80% (v/v) aq ethanol. These extracts were evaporated to dryness under reduced pressure at 35° and extracted with water to give the water-soluble fractions which were then divided into basic, neutral and acidic fractions by ion-exchange chromatography as described previously [14] The sugars in the neutral fractions were separated by paper chromatography in ethyl acetate–pyridine–water (8 2·1)

Measurement of Radioactivity

The radioactivity of eluates from paper chromatograms was determined from samples dried onto metal planchettes All other 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]

[18] A S. Spirin and L P. Gavrilova, *The Ribosome*, Springer-Verlag, Berlin (1969).
[19] I R MacDonald, J. S. D. Bacon, D Vaughan and R. J Ellis, *J Exptl Botany* **17**, 822 (1966)
[20] P. Siekevitz, *J. Biol Chem* **195**, 549 (1952).
[21] T ap Rees, E Blanch and D. D Davies, *Plant Physiol* **40**, 748 (1965).

Measurement of Oxygen Uptake

Oxygen uptake of samples of 25 disks in 2·5 ml medium was determined manometrically at 25° by Warburg's direct method. Unless stated otherwise, the disks were suspended in 0·02M KH_2PO_4 (pH 5·2) or in solutions of inhibitors dissolved in 0·02M KH_2PO_4 at pH 5·2.

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