Phytochemistry, 1968, Vol. 7, pp. 1453 to 1463. Pergamon Press. Printed in England

METABOLIC CHANGES IN EXCISED FRUIT TISSUE—II.

THE DEVELOPMENT OF A LIPID SYNTHESIS SYSTEM DURING THE AGEING OF PEEL DISKS FROM PRE-CLIMACTERIC APPLES

T. GALLIARD, M. J. C. RHODES, L. S. C. WOOLTORTON and A. C. HULME

Agricultural Research Council, Food Research Institute, Earlham Laboratory, Recreation Road, Norwich, Norfolk

(*Received* 31 *January* 1968)

Abstract—Disks of peel from immature (pre-climacteric) apples develop a lipid synthesizing system when aged aerobically at 25°. Full development of the system is rapid, occurring within 2–4 hr ageing and is dependent upon protein synthesis, being reversibly inhibited by low concentrations of cycloheximide. The ability to oxidize fatty acids and acetate to CO_2 is also increased by ageing. The nature of the lipids formed from ^{14}C-1-acetate by fresh and aged disks of apple peel is described.

INTRODUCTION

WHEN disks of underground storage tissues are incubated aerobically for several hours (a process commonly known as ageing) RNA and protein synthesis is stimulated in the disks, leading to the development of new enzyme systems.[1] Recent work from several laboratories has indicated that ageing of disks from storage tissues is associated with changes in membraneous components of cells, e.g. mitochondria,[2–4] and endoplasmic reticulum.[5]

The involvement of changes in membrane permeability and in the structure and properties of subcellular organelles in ripening processes in fruit has been the subject of recent studies.[6,7] Willemot and Stumpf[8,9] demonstrated the development of a fatty-acid synthesizing system during the ageing of disks of potato tuber and, since lipid metabolism would be expected to be associated with changes in lipoprotein membrane systems,we have extended our previous investigations[10,11] on changes occurring during ageing of disks from the peel of pre-climacteric apples to include the study of changes in lipid metabolism in this process.

RESULTS

Apple peel disks were aged routinely at 25° by aerobic incubation in a solution containing chloramphenicol to minimize bacterial contamination.[12] Lipid synthesis was assayed by

[1] R. E. CLICK and D. P. HACKETT, *Proc. Nat. Acad. Sci. U.S.* **50**, 243 (1963).
[2] S. G. LEE and R. M. CHASSON, *Physiol. Plantarum* **19**, 199 (1966).
[3] T. ASAHI, Y. HONDA and I. URITANI, *Plant Physiol.* **41**, 1179 (1966).
[4] R. F. M. VAN STEVENINCK and M. E. JACKMAN, *Australian J. Biol. Sci.* **20**, 749 (1967).
[5] M. E. JACKMAN and R. F. M. VAN STEVENINCK, *Australian J. Biol. Sci.* **20**, 1063 (1967).
[6] J. M. BAIN and F. V. MERCER, *Australian J. Biol. Sci.* **17**, 78 (1964).
[7] J. A. SACHER, *Plant Physiol.* **41**, 701 (1966).
[8] C. WILLEMOT and P. K. STUMPF, *Can. J. Botany* **45**, 579 (1967).
[9] C. WILLEMOT and P. K. STUMPF, *Plant Physiol.* **42**, 391 (1967).
[10] M. J. C. RHODES, T. GALLIARD, L. S. C. WOOLTORTON and A. C. HULME, *Phytochem.* **7**, 405 (1968).
[11] M. J. C. RHODES, L. S. C. WOOLTORTON, T. GALLIARD and A. C. HULME, **7**, 1439 (1968).
[12] C. J. LEAVER and J. EDELMAN, *Nature* **207**, 1000 (1965).

removing the disks from this solution, washing and then incubating with acetate-1-^{14}C. At the end of the incubation, the disks were killed and the lipids extracted for radioactivity counting or for further analysis.

Changes during Period of Ageing

Figure 1 illustrates a time course experiment in which the ability to incorporate acetate-1-^{14}C into lipid was measured after various times of ageing in both the control medium and in a medium to which was also added the protein synthesis inhibitor, cycloheximide (actidione), at a concentration of 1 μg/ml. The assays with labelled acetate-1-^{14}C were all performed in

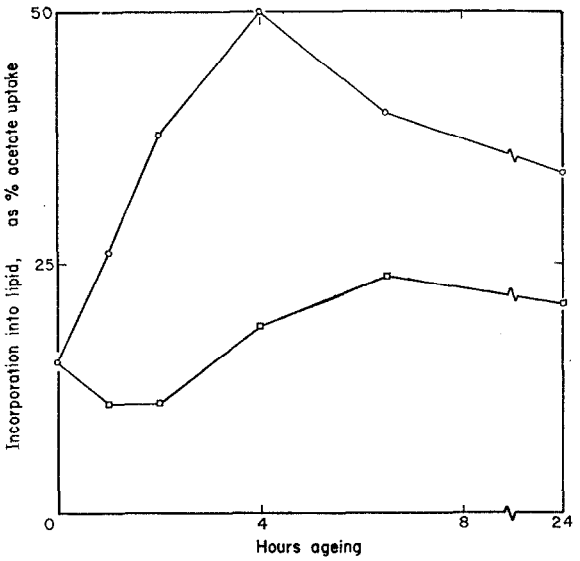

FIG. 1. INCORPORATION OF ACETATE-1-^{14}C INTO LIPID DURING AGEING OF APPLE PEEL DISKS.

Ability to incorporate acetate-1-^{14}C into lipid (expressed as per cent of acetate taken up which was recovered in the lipid fraction) was assayed in 1 hr incubations after different periods of ageing in the absence (○—○) and in the presence (□—□) of cycloheximide (1 μg/ml). Conditions of ageing are described in the text; the incubation system for the assay contained twenty disks (approx. 1 g fresh weight); potassium phosphate, 125 μmoles; sodium acetate-1-^{14}C, 1 μC (20 mμmoles) in a total volume of 2·5 ml at pH 4·5, incubated at 25° with shaking for 1 hr.

the absence of cycloheximide. It can be seen that the rate of incorporation of labelled acetate into lipid reached a maximal rate within 4 hr of ageing and that the maximal rate was 2·5 times* that in the fresh disks (i.e. assayed immediately without ageing). Cycloheximide inhibited the rapid rise in the rate of lipid synthesis during the first few hours of ageing, indicating that the observed rise in the control was dependent upon synthesis of new protein, presumably enzymes associated with lipid synthesis. With longer periods of ageing there is a partial recovery from the inhibitory effect of cycloheximide.

* The difference between the rate of synthesis in fresh disks and that in aged disks is greater in more immature fruit. As the fruit approaches the climacteric of development, the rate in fresh disks approaches that of aged disks.[13] Because of the seasonal nature of the fruit used, some experiments were necessarily performed with apples at later stages of development and in which initial rates were increased.

[13] A. C. HULME, M. J. C. RHODES, T. GALLIARD and L. S. C. WOOLTORTON, in preparation.

Properties of the Lipid Synthesizing System

The rate of uptake of exogenous acetate-1-^{14}C during incubation by disks previously aged for 6 hr is shown in Fig. 2 which also shows the rate of incorporation of acetate-1-^{14}C into lipid during the incubation. The high efficiency of the conversion of acetate to lipid is illustrated by the fact that after 90 min incubation, in which 86·5 per cent of the labelled acetate has been taken up by the disks, 52·5 per cent of the counts added as acetate-1-^{14}C (61 per cent of the counts taken up by the disks) was recovered in the lipid extract.

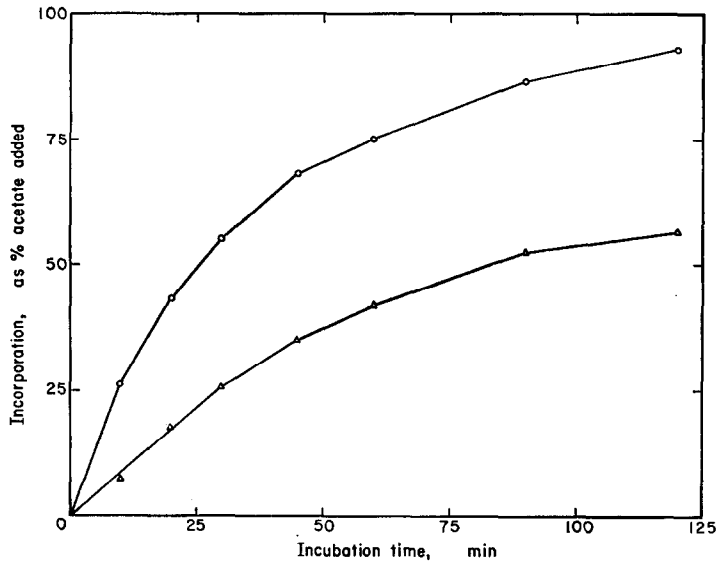

FIG. 2. UPTAKE AND INCORPORATION INTO LIPID OF ACETATE-1-^{14}C BY AGED DISKS OF APPLE PEEL.
Uptake of acetate-1-^{14}C (○—○) and incorporation into lipid (△—△), expressed as per cent of added acetate-1-^{14}C, was determined in incubations with disks which had previously been aged for 6 hr. The incubation system was as given in Fig. 1.

Evidence that the disks can convert substrate amounts of acetate to lipid with high efficiency is illustrated by an experiment (Table 1) in which amounts of non-radioactive

TABLE 1. EFFECT OF CONCENTRATION OF ACETATE ON ITS INCORPORATION INTO LIPIDS

Acetate added mμmoles	Acetate taken up		Acetate incorporated into lipid	
	mμmoles	%	mμmoles	As % of uptake
20	15	75	9	60
50	40	80	25	62·5
100	70	70	42	60
500	300	60	140	47
1000	540	54	210	39

The incubation system contained twenty disks (approx. 1 g fresh weight); potassium phosphate 125 μmoles; ^{14}C-1-acetate, 1 μC (20 mμmoles); sodium acetate as indicated in a total volume of 2·5 ml at pH 4·5. Incubation was at 25° for 1 hr.

sodium acetate were added to the labelled isotope in the incubation and then uptake and conversion of acetate to lipid was determined. The lipid synthesis system was not saturated up to 1 μmole of added acetate, at which level 54 per cent was taken up and 35 per cent of the acetate taken up was converted to lipid in 1 hr.

Effects of Inhibitor

The effects of inhibitors were studied in the two separate processes, i.e. (*a*) effects on the development of the lipid synthesis system on ageing and (*b*) effects on the incubation of aged disks with acetate-1-^{14}C.

TABLE 2. EFFECT OF CONDITIONS DURING AGEING ON THE INCORPORATION OF ACETATE-1-^{14}C INTO LIPIDS

Conditions during ageing	Period of ageing (hr)	Lipid formed from acetate-1-^{14}C as % of uptake
Expt. 1		
Fresh disks	0	25
Control	24	45
Atmosphere of 3% O_2–97% N_2	24	46
+Chloramphenicol (2 mg/ml)	24	17
+Puromycin (100 μg/ml)	24	46
+6-Azauracil (100 μg/ml)	24	44
Expt. 2		
Fresh disks	0	41
Control	3	60
Control	24	54
+Cycloheximide (0·1 μg/ml)	3	40
+Cycloheximide (0·1 μg/ml)	24	38
+*p*-Fluorophenylalanine (2 mg/ml)	3	71
+*p*-Fluorophenylalanine (2 mg/ml)	24	33
+Actinomycin D (50 μg/ml)	3	53
+Actinomycin D (50 μg/ml)	24	52

Ageing conditions described in text. Incubations as described in Fig. 1.

The effects on development were examined by performing the ageing process in the presence of inhibitors then washing the disks before incubating with acetate-1-^{14}C, in the absence of inhibitor.

Table 2 lists the effects of inhibitors studied. Of all the protein synthesis inhibitors used, only cycloheximide (even at the very low concentration of 0·1 μg/ml), chloramphenicol (2 mg/ml) and *p*-fluorophenylalanine (2 mg/ml) prevented the increased lipid synthesis after 24 hr ageing. The effect of *p*-fluorophenylalanine is not directly on the development of the lipid synthesis system since it did not inhibit the rise observed after a 3-hr ageing period and in most cases showed a slight stimulation of the effect over a short period. The concentrations of all inhibitors used were those which prevent the rise in other processes (e.g. malate decarboxylation[11]) on ageing. Chloramphenicol at the concentration routinely used (50 μg/ml) to minimize bacterial growth during ageing had no effect on the development of lipid synthesis.

Although cycloheximide (0·1 μg/ml) inhibits the development in lipid synthesis during ageing, it has no inhibitory effect (at 1 μg/ml) on the incorporation of acetate-1-^{14}C into lipids in disks which have been allowed to age in the absence of inhibitor (Table 3).

TABLE 3. COMPARISON OF THE EFFECTS OF CYCLOHEXIMIDE (CH) DURING AGEING AND INCUBATION ON THE INCORPORATION OF ACETATE-1-^{14}C INTO LIPID

Conditions	Lipid formed as % of acetate uptake
Fresh disks	44
Aged −CH incubated −CH	64
Aged −CH incubated +CH (1·0 μg/ml)	65
Aged +CH (0·1 μg/ml) incubated −CH	39

Ageing period 3 hr. Incubation period 1 hr. Conditions as described in Fig. 1.

That the inhibitory effect of cycloheximide during ageing is reversible, is shown in an experiment (Fig. 3) in which disks were aged in cycloheximide for 4 hr (during which time control disks aged without inhibitor had developed maximal lipid synthesis) and then aged in a medium free of inhibitors; the disks recovered to the control level within a further 8 hr ageing.

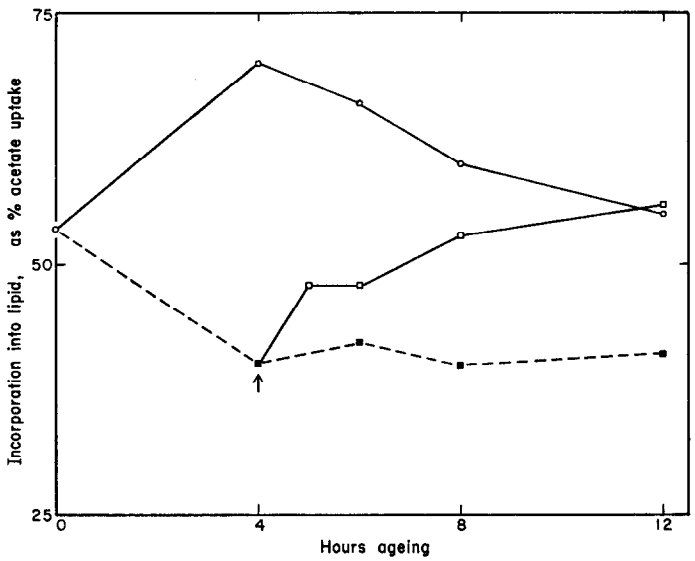

FIG. 3. REVERSIBLE INHIBITION BY CYCLOHEXIMIDE OF THE DEVELOPMENT OF A LIPID SYNTHESISING SYSTEM DURING AGEING OF APPLE PEEL DISKS.

Incorporation of acetate-1-^{14}C into lipid was assayed in disks which had been aged; (*a*) in the absence of cycloheximide (○—○), (*b*) in the presence of cycloheximide, 1 μg/ml (■---■), and (*c*) in the presence of cycloheximide for 4 hr before transfer to a medium without cycloheximide (□—□). The incubation system was as described in Fig. 1.

Uptake of Acetate-1-^{14}C and Conversion to $^{14}CO_2$

Figure 4 illustrates the time course of $^{14}CO_2$ evolution in disks incubated with acetate-1-^{14}C for 1 hr after periods of ageing. There is a gradual increase in the rate of production of $^{14}CO_2$ during the first 24 hr of ageing, in contrast to the lipid synthesis system in which a maximal rate is attained within 4 hr. The effects of protein synthesis inhibitors during ageing on the rate of CO_2 production were similar to those in the lipid system, cycloheximide again inhibiting the increase during ageing. The rate at which sodium acetate-1-^{14}C is taken up by the disks from external solution increases only slightly on ageing (Fig. 4).

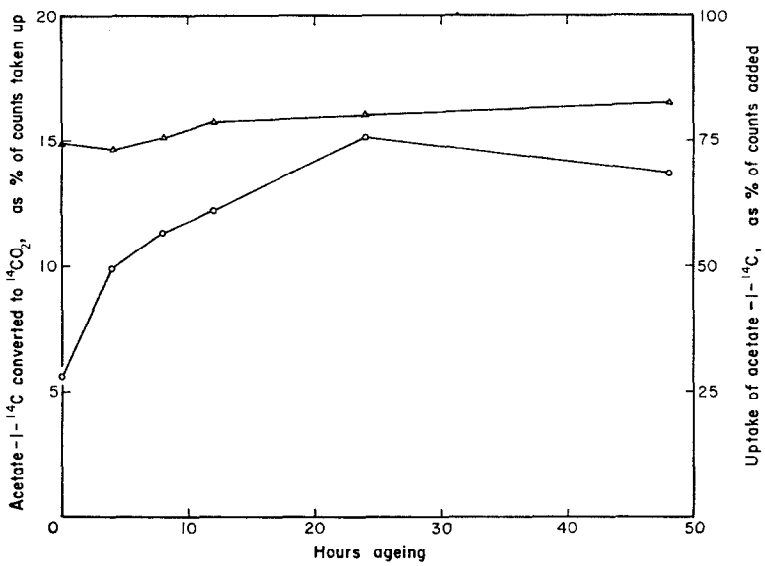

FIG. 4. UPTAKE AND CONVERSION TO $^{14}CO_2$ OF ACETATE-1-^{14}C DURING AGEING OF APPLE PEEL DISKS. Uptake (△—△) and conversion to $^{14}CO_2$ (○—○) were determined in 1 hr incubations after periods of ageing. The incubation conditions were as given in Fig. 1.

TABLE 4. EFFECT OF AGEING ON UPTAKE AND $^{14}CO_2$ PRODUCTION FROM LINOLEIC ACID-1-^{14}C

	Linoleic acid-1-^{14}C taken up* (%)	$^{14}CO_2$ produced as % of linoleic acid uptake
Fresh disks	50	3
Aged 24 hr	57	8
Aged 24 hr in presence of cycloheximide (1 μg/ml)	56	4

* The amount of linoleic acid-1-^{14}C taken up by the disks represents counts disappearing from the incubation medium and not recoverable from the disks by a "cold wash" at 0° with non-radioactive 1 mM linoleic acid.

Ageing conditions were as described in Experimental. The assay system contained twenty disks, potassium phosphate (125 μmoles), linoleic acid-1-^{14}C (NH_4^+ salt), 1 μC (20 mμmoles) in volume of 2·5 mls at pH 4·5. Incubation at 25° for 1 hr.

Changes in Fatty Acid Oxidation Capacity During Ageing

To examine the possibility that fatty acid oxidation, in addition to synthesis, is stimulated during ageing and thus reflecting a general increase in fatty acid turnover, fresh and aged disks were incubated with linoleic acid-1-^{14}C and the $^{14}CO_2$ produced was measured. Parallel measurements of the uptake of linoleic acid were performed. The results shown in Table 4 indicate an increased rate of $^{14}CO_2$ production (expressed as per cent of linoleic acid taken up by the disks) on ageing. The increase was prevented by ageing in the presence of cycloheximide, again indicating the involvement of protein synthesis in the development of a fatty acid oxidation system and thus of fatty acid turnover.

Studies on Other Lipid Precursors

The results given above demonstrate an increase on ageing of the ability of disks to synthesize and oxidize the fatty acid components of lipids. Experiments with labelled acetate do not distinguish between increased turnover of the fatty acid components of pre-existing lipids and increased synthesis of lipids *de novo*. To obtain information on this, glycerol-1-^{14}C and orthophosphate-^{32}P were tested as substrates for incorporation into the glycerol moiety of glycerolipids and the phosphate group of phospholipids respectively.

However, both glycerol and inorganic phosphate are not taken up readily from solution by fresh disks. The ability to take up these materials increases markedly during ageing[11] thus complicating studies on relative increases in lipid synthesis from these precursors.

Inorganic phosphate-^{32}P is taken up to the extent of approximately 5 per cent per hour from a 10^{-5} M solution by fresh disks and less than 1 per cent of the amount taken up is incorporated into phospholipid.

Glycerol-1-^{14}C was used in preliminary experiments and again a marked increase of uptake subject to inhibition by cycloheximide was observed on ageing the disks. Incorporation into lipid was variable but no marked increase in incorporation into lipid (when expressed as percentage of glycerol taken up) was observed on ageing.

Identification of Lipids Labelled from Acetate-1-^{14}C

To investigate the nature of labelled lipids, fresh and aged disks were incubated with acetate-1-^{14}C (25 μC). The extracted lipids were examined (*a*) for labelled fatty acid composition by radio–gas chromatography of methyl esters, (*b*) for total lipid labelling by two-dimensional thin-layer chromatography of the lipid extract, followed by autoradiography and removal of lipids from the thin layer for counting.

To aid identification of the radioactive spots on the autoradiographs, an aliquot of non-radioactive lipid extract of known composition[14] from potato was added as marker for the thin-layer separation. The identity and radioactivity counts of individual lipids from the disks are shown in Table 5.

Approximately 60 per cent of the counts in fresh disks were recovered in phospholipids, phosphatidylcholine being most heavily labelled, phosphatidylglycerol and polyglycerophosphatide were labelled more than phosphatidylinositol or phosphatidylethanolamine, no labelling of phosphatidic acid was observed. The neutral (non-polar) lipids comprise about 30 per cent of the counts, the remainder being in a spot which runs on the thin-layer plate coincident with free fatty acids. With aged disks, although there is an increase in the counts of most components, there is a large increase in the counts incorporated into "free fatty acid". The labelling of lipids in disks aged in cycloheximide is similar to that in fresh disks.

[14] T. GALLIARD, *Phytochem.*, in press.

TABLE 5. INCORPORATION OF ACETATE-1-^{14}C INTO INDIVIDUAL LIPIDS OF FRESH AND AGED DISKS

Identity	Counts in each lipid as % of total lipid counts		
	Fresh	Aged control (%)	Aged in cycloheximide
Phosphatidylinositol	4·2	3·1	2·8
Phosphatidylcholine	34·1	14·5	22·6
Digalactolipid	1·0	1·0	1·0
Phosphatidylglycerol	6·7	4·2	10·6
Phosphatidylethanolamine	3·4	3·1	2·8
Free fatty acid	11·6	43·2	20·3
Polyglycerophosphatide	10·3	10·1	9·8
Neutral lipid	28·7	20·8	31·0
Total activity (=100%) d/100s × 10^{-6}	10·9	26·3	10·8

Ageing conditions are described in the text. The incubations were as described in Fig. 1 except that sodium acetate-1-^{14}C (25 μC, 570 mμmoles) was added as substrate.

The nature and composition of the "free fatty acid" fraction which incorporates the majority of counts in the aged disks is under further study. Polyglycerophosphatide, although a minor component of the lipids of apple peel, incorporates a considerable percentage of the counts and thus would show a high specific radioactivity. (A similar result is obtained when aged disks of potato tissue are incubated with acetate-1-^{14}C and inorganic phosphate-^{32}P (unpublished results).)

Fatty acid methyl esters, prepared from total lipid extracts by transmethylation, contained all the radioactivity initially present in the total lipids formed from acetate-1-^{14}C. Preliminary analyses by argentation thin-layer chromatography and radio–gas chromatography of the radioactive fatty acid methyl esters gave the following percentage distribution of label with fresh disks incubated 1 hr with acetate-1-^{14}C: palmitic acid (21·8 per cent), stearic acid (11·0 per cent), oleic acid (54·5 per cent), linoleic acid (12·7 per cent), other fatty acids contained only negligible amounts of radioactivity. Parallel experiments with disks aged for 24 hr showed increased incorporation into saturated acids; palmitic (35·5 per cent), stearic (18·7 per cent), less incorporation into oleic acid (45·8 per cent) and no incorporation into linoleic acid was obtained. No incorporation was observed into linolenic acid or long-chain fatty acids with either fresh or aged disks.

DISCUSSION

The development of a lipid synthesizing system during ageing of disks from immature apple peel is one aspect of a series of changes, dependent on protein synthesis, which are initiated by excision. The ageing period required to develop maximal effect varies; of those studied so far[10, 11, 15] lipid synthesis is the most rapid (2–4 hr ageing).

Most work on ageing of disks has been concentrated on storage organ tissues. Willemot and Stumpf[8, 9] have shown that, during ageing of disks of potato tuber, there is a marked

[15] T. GALLIARD, M. J. C. RHODES, L. S. C. WOOLTORTON and A. C. HULME, *Phytochem.* **7**, 1465 (1968).

increase in fatty acid synthetase activity dependent upon protein and RNA synthesis. Our work confirms that a similar process occurs during the ageing of disks from immature fruit. The effect in apple reaches a maximal level within a shorter time at 25° than in potato and the capacity to synthesize fatty acids from acetate-1-^{14}C is also greater in apple.

Whether the increased lipid synthesis in aged disks represents an increase in total lipid synthesis, or is restricted to the fatty acid moieties of lipids, has not yet been conclusively ascertained. Preliminary results with glycerol-1-^{14}C indicate that this precursor is not incorporated into lipid at an increased rate in aged disks. As outlined below, there may also be a spatial limitation of sites of synthesis of lipid constituents within the cells. Exchange of fatty acids on existing lipids could also be a factor in the increased labelling of lipids in aged disks.[16]

Of the protein synthesis inhibitors tested, only low concentrations of cycloheximide and relatively high amounts of chloramphenicol prevented the development of lipid synthesis during ageing. Willemot and Stumpf[9] showed that cycloheximide inhibited the increased lipid synthesis in aged potato disks but that actinomycin D showed a variable and delayed inhibitory effect and puromycin had only a partial effect. In our work with apple disks, neither actinomycin D nor puromycin inhibited the development. In the development of the malate decarboxylation system the increase on ageing was completely blocked by the above inhibitors and also by *p*-fluorophenylalanine and azauracil. The explanation of the discrepancy in the effects of these inhibitors may be in the intracellular loci of these effects. Malic enzyme (E.C. 1.1.1.40) is known to be a cytoplasmic enzyme; fatty acid synthesis from acetate requires activation of acetate to acetyl coenzyme A and Yang and Stumpf[17] have shown that in higher plants acetyl thiokinase does not occur in the cycloplasm but is restricted to the mitochondria. If the development of the fatty acid synthesizing system was confined to a particulate fraction inaccessible or insensitive to inhibitors of cytoplasmic protein synthesis, the differences in inhibitor response could be explained. Since the lipid synthesis reaches a maximum after a relatively short period of ageing, there may also be an initial period of ageing in the presence of some inhibitors during which the inhibitors have not reached an effective concentration at their sites of action. Although there is no direct information on these aspects, evidence from studies with ageing of potato disks showed that in pulse-chase experiments, most of the lipid labelled from acetate-1-^{14}C in short time periods was associated with a mitochondrial fraction after centrifugation (unpublished results).

Several workers have implicated changes in structure and number of cell organelles during ageing in disks of storage tissues.[2–5] Such changes would involve the lipid moieties of membrane systems and thus examination of those lipids turning over during ageing was important.

In fresh disks phospholipids, particularly phosphatidylcholine, were rapidly labelled from acetate-1-^{14}C and this group of lipids is known to be an important component of many membrane systems. A similar accumulation of counts from acetate-1-^{14}C into phosphatidylcholine was observed in peel tissue from ripe apples.[24] Galactolipids, important components of plastid membranes, were not labelled from acetate in our experiment. Although the total counts in all lipids are increased in aged disks incubated with acetate-1-^{14}C, the much larger increase in incorporation into a lipid with the chromatographic properties of free fatty acid indicates an uncoupling of integrated lipid synthesis during ageing. The changes in the pattern of fatty acid synthesis during ageing of apple peel disks does not appear to be as marked

[16] W. E. M. Lands, *J. Biol. Chem.* **235**, 2233 (1960).
[17] S. F. Yang and P. K. Stumpf, *Biochim. Biophys. Acta* **98**, 19 (1965).

as that observed during ageing of potato disks.[8,9] However, significant differences from the potato system were obtained, particularly in the fall in the proportion of unsaturated fatty acids incorporating labelled acetate in aged disks of apple peel.

Evidence that effects observed during ageing in these experiments are due to changes in the apple tissue, and not to microbial contamination, is summarized in the previous paper.[11]

EXPERIMENTAL

Materials

Cox's Orange Pippin apple fruits were picked from trees growing on Malling IX root stocks at Burlingham Horticultural Station, Norfolk. Apples used for the present experiments were all in the pre-climacteric state and were used after monitoring the respiration of the whole fruits[18] in chambers maintained at 12°.

Chemicals were obtained as follows: chloramphenicol, B.P. (Boots Pure Drug), puromycin and cycloheximide (Sigma), *p*-fluorophenylalanine (British Drug Houses), 6-azauracil (CalBiochem); actinomycin D was a gift from Merck, Sharp and Dohme (Rahway, New Jersey).

Sodium acetate-1-^{14}C (44·4 μC/μmole), glycerol-1-^{14}C (23·6 μC/μmole), linoleic acid-1-^{14}C (53·3 μC/μmole) and orthophosphate-^{32}P (carrier-free) were purchased from the Radio Chemical Centre, Amersham, Bucks.

Methods for Ageing and Incubating Disks

The method of preparation of apple peel disks was described earlier.[10] Ageing of disks was carried out by gentle shaking at 25° in conical flasks with 0·05 M phosphate, pH 4·5, containing chloramphenicol (50 μg/ml) and, where indicated, inhibitors etc. Approximately 1 ml solution was used per disk. The solutions were changed after 1 hr and at intervals during the ageing period. Disks which were to be incubated with inorganic^{32}P were treated differently in that phosphate buffers were omitted during disk preparation and ageing, the pH being adjusted to pH 4·5.

Before incubation for assay of lipid synthesis etc., disks were washed with distilled water and lightly blotted. All incubations were at 25° in 25-ml conical flasks placed in a shaking bath. Routinely twenty disks (approximately 1 g fresh weight) were incubated in 2–3 ml 0·05 M potassium phosphate containing substrate and additions as indicated in the text.

$^{14}CO_2$ evolution was determined as described previously.[11] To measure radioactivity in lipids formed from acetate-1-^{14}C and glycerol-1-^{14}C, the incubation solution was decanted from the disks which were then repeatedly washed with water. Washings and decanted solutions were combined to measured volume and an aliquot counted for uptake determination. Disks incubated with linoleic acid-1-^{14}C were washed at 0° with 10^{-3} M linoleic acid (non-radioactive) for 30 min following the water wash, to remove any adsorbed and "free space" substrate. Carrier potassium phosphate was added to incubation media containing orthophosphate-^{32}P so that the phosphate concentration was 10^{-5} M; after incubation and water washing, the disks were washed at 0° with non-radioactive potassium phosphate 0·1 M, pH 4·5, before extracting lipids.

Lipid Extraction

Rapid killing of disks was important in avoiding production of artefacts by enzyme action.* The following method based on the lipid extraction technique of Bligh and Dyer[19] was satisfactory and minimized transfer losses.

After washing, twenty disks were lightly blotted and dropped into a 2·8 cm dia. boiling tube fitted with an air condenser and containing refluxing aqueous methanol—10 ml and the refluxing continued for 5 min. The mixture was cooled and $CHCl_3$ (4 ml) added. The disks were homogenized in the tube with an Ultra Turrax homogenizer (Model TP18/2, Janke+Kunel KG. Staufen, Germany). The homogenizer shaft was washed by inserting in a clean tube containing 7·6 ml of $CHCl_3$–methanol–water (4:2:1·6 v/v/v) and adding the washing to the homogenate which now had the single phase composition of Bligh and Dyer.[19] The lipids in $CHCl_3$ solution were obtained by pouring the mixture through glass wool into a separating funnel, containing 1 per cent sodium acetate in 2M KCl (pH 7·5)—6 ml. The boiling tube and glass wool were washed with $CHCl_3$ (6 ml) which was added to the mixture and then shaken to give a clear lower $CHCl_3$ phase, containing

* Disks of potato tuber incubated with acetate-1-^{14}C and orthophosphate-^{32}P produce large amounts of labelled phosphatidic acid (by phospholipase D activity) if the lipids are extracted by adding chloroform–methanol (2:1 v/v) to disks and then rapidly heated to 68° (unpublished results). No phosphatidic acid is obtained if the disks are placed in refluxing aqueous methanol.

[18] A. C. HULME, J. D. JONES and L. S. C. WOOLTORTON, *Proc. Roy. Soc., Lond.* Series B, **158**, 519 (1963).
[19] E. G. BLIGH and W. J. DYER, *Can. J. Biochem. Physiol.* **37**, 911 (1959).

negligible amount of water-soluble counts. Aliquots of the $CHCl_3$ solution were evaporated and dissolved in scintillation mixture for counting. For further separation and analysis, the $CHCl_3$ solutions were evaporated *in vacuo*, and the residue dissolved in benzene–ethanol (4:1 v/v) containing 5 mg per cent butylated hydroxy toluene (BHT) and stored under N_2 at −20° until analysed.

Analysis of ^{14}C-Labelled Lipids

Aliquots of lipid solutions containing 5–10 mμC ^{14}C-labelled lipid together with an aliquot of potato lipid of known composition[14] in benzene–ethanol solution, were separated by two-dimensional TLC on 20 × 20 cm glass plates coated with a 250 μ layer of Silica Gel G (Merck), using a system similar to Nichols.[20] $CHCl_3$–methanol–7 N ammonium hydroxide (65:25:4 v/v/v) containing 5 mg per cent BHT[21] was used to develop the plate in the first dimension. The plates were re-activated by heating at 50° for 30 min in a vacuum oven, O_2-free N_2 was used to release the vacuum and the plate developed in the second dimension in $CHCl_3$–methanol–glacial acetic acid–water (170:25:25:4). Thin-layer plates were warmed to remove acetic acid which otherwise causes darkening of X-ray film during autoradiography.

Autoradiography of developed TLC plate was performed with Industrial-G X-ray film (Ilford) for 4–7 days. Areas on the plate corresponding to darkened areas on the developed X-ray film were marked, the plate exposed to iodine vapour to identify positions of known components. After removing the iodine, radioactive areas were scraped into vials for counting.

Analysis of ^{14}C-Labelled Fatty Acids

Aliquots of ^{14}C-labelled lipid in 5 per cent methanolic HCl were heated in stoppered tubes for 8 hr at 65°. After cooling, and dilution with water, methyl esters were extracted into hexane and stored under nitrogen in benzene containing 5 mg per cent butylated hydroxy toluene. Thin-layer separations were performed as above on silica gel containing 3 per cent (w/w) silver nitrate and developed in hexane–diethyl ether, 9:1 (v/v) prior to autoradiography. Radio–gas chromatographic separation of the fatty acid methyl esters was performed by the method of James and Piper[22] using diethylene glycol adipate as stationary phase.

Radioactive Counting

Liquid scintillation counting (Panax Instruments Ltd, Redhill, Surrey) was used. For scintillation mixtures based on Triton X-100:toluene solutions,[23] a stock solution was prepared by mixing 1 volume of Triton X-100 (Rohm and Haas Inc.) and 2 volumes of a mixture of 4 g PPO (diphenyl oxazole) and 0·1 g POPOP (1,4-bis-2-(5-phenyloxazolyl)-benzene) in 1 l. of toluene.

Lipid samples were dissolved in the stock solution for counting at 70 per cent efficiency for ^{14}C. Aqueous samples were counted at 60 per cent efficiency in mixtures containing 1 volume of water and 9 volumes of stock solution. Radioactive areas from thin-layer plates were counted by suspending the silicic acid powder in a gel mixture containing 250 mg Cab-O-Sil (Packard Instruments Ltd.), 0·3 ml water (to deactivate the silicic acid) and 5 ml dioxan scintillation mixture (PPO, 7 g; POPOP, 0·3 g; naphthalene, 100 g; made to 1 l. with dioxane). 90–100 per cent of the counts applied to the thin layer were recovered in this system. Duplicate thin-layer plates were run and blanks obtained by taking non-radioactive areas through the above procedure.

Acknowledgements—Mr. A. S. A. Pryke gave excellent technical assistance. We are indebted to Dr. M. I. Gurr of the Unilever Research Laboratories, Colworth House, for performing radio–gas chromatographic analyses, and to Mr. P. Harkett and Miss S. Bugg for preparation of figures.

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