Fat Metabolism in Higher Plants. XXXIV. Development of Fatty Acid Synthetase as a Function of Protein Synthesis in Aging Potato Tuber Slices

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Summary. Experiments with inhibitors of protein synthesis (actinomycin D, puromycin, actidione) showed that the increase and the change in fatty acid synthetase activity, observed during the aging of potato disks, were accompanied by and related to a temporary rise in the rate of protein and RNA synthesis. These results concur with the earlier suggestion by Click and Hackett that the aging process involves a type of derepression. A possible course of events during aging, and possible derepression mechanisms are suggested and discussed.

In a recent paper (26), we described the development of fatty acid synthetase activity in aging potato tuber tissue. During the first hours of the aging process, the fatty acid synthetase activity of potato disks increases 6 to 12-fold. During the same period, the tissue synthesizes increasing amounts of poly-unsaturated (especially linolenic acid) and long chain (Ca25-C27) fatty acids, which are not synthesized by the disks immediately after slicing. The quantitative and qualitative changes in the synthesis of fatty acids occur very early during the aging process, earlier than the changes in respiration described by Laties (10-12) in the same tissue under the same conditions of aging. This observation makes difficult a correlation of the sudden rise in metabolic activity with a relief from a negative feedback control of respiration, determined by the level of a volatile intermediate (11).

Click and Hackett (4) demonstrated that the changes occurring during the aging process depended on the synthesis of new RNA and protein, and suggested "that release from some respiratory inhibition will not per se account for the observed changes." However, Marré and his collaborators (17) found that in the very early steps in the germination of the castor bean, 2 processes appear to occur, namely, a reactivation of pre-existing enzymes as well as de novo synthesis of enzyme proteins.

Because of the very early rise in fatty acid synthetase activity in the aging potato tissue, it was of interest to determine whether this aspect of the reactivation of metabolism also depended on the synthesis of new protein.

The present paper demonstrates that the rise in fatty acid synthetase activity during the aging of potato tuber tissue relates to a temporary rise in the rate of protein and RNA synthesis.

Materials and Methods

Most materials and methods used in this work have been described previously (26).

Acetate-1-14C (55.8 mc/mimole), L-leucine-UL-14C (275 mc/mimole), L-lysine-UL-14C (247 mc/mimole), uridine-2-14C (40.6 mc/mimole) were purchased from Nuclear Chicago. Actidione (cycloheximide) and puromycin were obtained from the Nutritional Biochemicals Corporation, chloromycetin from Parke Davis and Company, some puromycin from Sigma, and some actinomycin D from Mann Laboratories. Most of the actinomycin D used in this work was generously given to us by Dr. Arnold Demain of Merck-Sharp and Dohme.

The potato disks were aged by shaking at room temperature (24°-26°) in an aqueous solution of 0.1 mm CaSO4 and chloromycetin (50 μg/ml). Under these conditions, no bacterial contamination was observed.

All assays were carried out by shaking 10 potato disks at room temperature in 1 ml of a solution containing 0.1 mm CaSO4, chloromycetin (50 μg/ml), 0.1 mm potassium phosphate buffer pH 5.5 and generally from 0.5 μe to 2 μe of labelled substrate.
All results are given as percentage of radioactivity supplied in the medium (in most cases from 0.5 μc to 2 μc, or approximately 1,000,000 to 4,000,000 cpm on a scintillation counter) to allow for easier comparison between experiments.

The rate of fatty acid synthesis was determined at different times of aging by measuring the incorporation of acetate-1-14C into the lipids of potato disks during a 2-hour incubation.

The methods selected for measuring the radioactivity in protein and RNA are based on those published by Laszlo and coworkers (9), Laties (13), Mans and Novelli (16), and Click and Hackett (4).

The rate of protein synthesis was determined by measuring the incorporation of leucine-UL-14C into the insoluble fraction of the disks during a 2-hour incubation. The reactions were stopped by the addition of 10 ml cold 10% trichloroacetic acid containing unlabelled leucine (0.2 mg/ml). The disks were allowed to stand overnight, then extracted twice for 30 minutes with cold 10% trichloroacetic acid, 3 times for 30 minutes with chloroform-methanol (2:1, v/v), and 3 times with boiling 70% ethanol containing unlabelled leucine. The disks were rinsed twice for 10 minutes with 95% ethanol, then dried at 80°. They were finally submerged in 10 ml of toluene scintillator and counted when all disks had settled. The same method was followed when the slices were incubated in the presence of lysine-UL-14C.

The rate of RNA synthesis was likewise determined by measuring the incorporation of uridine-2-14C into the insoluble fraction of the disks during a 3-hour incubation.

In the experiment where the rate of respiration was determined, the oxygen consumption was measured in a Gilson Differential Respirimeter. Series of 10 disks were aged in Warburg flasks shaken at 25°, in 2 ml of aging solution. Their respiration rate was continuously measured. Other disks were aged as described, and assayed for their ability to synthesize proteins and lipids. Preliminary experiments showed that at all times of aging the rates of respiration and of lipid synthesis were approximately the same in all series of disks, whether aged on the shaker or aged in the respirometer.

Results

The antibiotics actidione and actinomycin D, which specifically inhibit protein synthesis, also inhibit the observed rise in fatty acid synthetase activity during the aging of potato disks.

Actidione inhibits the translation step in protein synthesis by preventing the formation of the peptide bond at the level of the ribosomes (6). As indicated in figure 1, when added to the aging medium at a concentration of 2 μg per ml, actidione completely prevented the development of fatty acid synthetase activity. No inhibitor was added to the assay system. However, the antibiotic only partially inhibited the increase in the formation of 14CO₂ from acetate-1-14C during the assays. Puromycin, a widely used inhibitor which inhibits the same step in protein synthesis by acting as an analog of amino acyl tRNA (5, 7, 20), achieved only partial inhibition of the rise in fatty acid synthetase, when added to the aging medium at a concentration of 25 μg per ml.

Fig. 1. Inhibition by actidione of the development of fatty acid synthetase in aging potato tuber slices (1% = 35,500 cpm).

Fig. 2. Inhibition by actinomycin D of the development of fatty acid synthetase in aging potato tuber slices (1% = 15,000 cpm).
Actinomycin D (25 μg/ml), which blocks the transcription of DNA into messenger RNA (8), also inhibited the development of fatty acid synthetase during aging, but to a lesser extent than actidione (fig 2). The degree of inhibition by actinomycin D varied markedly. In most experiments, inhibition was preceded by a lag period of up to 8 hours, during which the inhibitor actually stimulated the development of fatty acid synthetase (fig 3A and 4A). However, in all experiments, actinomycin D inhibited the rise in the formation of 14CO2 from acetate-1-14C to the same extent.

Figure 3 illustrates the results of an experiment where potato disks were aged in the presence or absence of actidione and actinomycin D. At various times of aging, some of the disks were withdrawn from the aging solution and assayed for their ability to synthesize fatty acids (fig 3A) and protein (fig 3B). In absence of inhibitor, fatty acid synthesis was greatly enhanced during the 11-hour period of the experiment. Protein synthesis, which was essentially inactive in the fresh tissue, increased markedly and reached a peak after 8 hours of aging. Actidione completely inhibited the development of both activities. Actinomycin D did not markedly affect the synthetase activity at the beginning of the aging period, but after 5 hours in the presence of the antibiotic, some inhibitory effect was observed. As with actidione, almost complete inhibition by actinomycin D was noticed with leucine incorporation into proteins.

Figure 4 shows the results of a similar experiment where the disks were assayed at different times of aging for their ability to synthesize either fatty acids (fig 4A) or RNA (fig 4B). The rate of fatty acid synthetase greatly increased during the first hours of aging, reaching a peak after 12 hours, and subsequently decreasing to a lower level which was then maintained. The rate of 14CO2 evolution continued to increase throughout the experiment. The incorporation of uridine-2-14C into the insoluble fraction of the disks, presumably RNA, increased 3-fold, and reached a peak after 6 hours of aging. However, within 2 hours, actinomycin D almost completely inhibited RNA synthesis. Actidione also inhibited RNA synthesis, but with a slight delay.

To determine whether de novo protein and RNA synthesis were required throughout the aging

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**Fig. 3.** Relation of the development of fatty acid synthetase (A) (1% = 31,000 cpm) with protein synthesis (B) (1% = 7000 cpm) in aging potato tuber slices.

**Fig. 4.** Relation of the development of fatty acid synthetase (A) (1% = 9000 cpm) with RNA synthesis (B) (1% = 8500 cpm) in aging potato tuber slices.
process, the inhibitory effect of actidione and actinomycin D on the development of fatty acid synthetase activity was tested, either by exposing the disks to the inhibitors after given periods of aging in the absence of inhibitors, or by exposing the disks to the inhibitors for varying periods of aging and then washing and returning the disks to an aging solution free of inhibitors (fig 5 to 8).

When actidione was added to the aging solution after 1 hour of aging (fig 5), synthetase activity was maintained at the level reached at the time of addition. However, when the disks were ex-

![Diagram](https://www.plantphysiol.org)
posed to actidione after 3 or 6 hours of aging, consistently a slight decrease in rate of fatty acid synthetase appeared, which was then reversed with further aging despite the presence of actidione in the medium.

When actidione was withdrawn from the aging solution after different times of aging in the presence of the inhibitor (fig 6), an increasing delay in the reversal of inhibition was observed with increasing time of exposure of the tissue to the inhibitor. Interestingly, practically complete recovery from the effects of actidione was eventually observed when the tissue was exposed for 1 or 3 hours to the inhibitor and then removed from the medium containing the inhibitor.

The recovery of the tissue from exposure to actidione is most pronounced in the experiment summarized in figure 6. It would appear that modifications of actidione are taking place in the plant cell thereby decreasing the inhibitory effects of actidione.

When actinomycin D was added to the aging solution after different times of aging (fig 7), the effects resembled those obtained with actidione (fig 5). Not only did the inhibitor prevent further development of fatty acid synthetase, but it also delayed the decrease in synthetase activity once the peak was reached, after 10 hours of aging. No reversal effect is observed in this experiment in contrast with a similar experiment using actidione (fig 5).

In the 3 experiments illustrated in figures 5 to 7, the inhibitory effects of actidione and actinomycin D on the formation of \( ^{14} \text{CO}_2 \) from acetate-1-\( ^{14} \text{C} \) were even more pronounced than their effects on the development of synthetase activity during aging. Figure 8 shows the effect of the delayed withdrawal of actinomycin D from the aging medium on the increase of \( ^{14} \text{CO}_2 \) evolution during the 2 hour assays. Here, however, the effect was in many respects the reverse of the one obtained with actidione : with longer exposure of the tissue to actinomycin D, the delay in recovery from the inhibition increased, while the rate of recovery decreased.

Parallel with the ability of the tissue to incorporate leucine after various times of aging, its ability to incorporate lysine was investigated (fig 9). The distinct peak in the rate of incorporation, which was observed 3 hours after the tissue was sliced, was confirmed in 3 other experiments. The interpretation of these results is difficult to assess at this time.

The time relationships of respiration, incorporation of lysine and leucine into the insoluble fraction of the disks, and incorporation of acetate into the lipids, are also depicted in figure 9. The various assays, carried out in the course of 1 experiment, used disks from the same batch of aging potato tissue.

![Fig 9](image-url)

**Fig. 9.** Time relationships of the rate of respiration (oxygen consumption, \( \mu \text{L/hr} \)), the rate of incorporation of lysine-UL-\( ^{14} \text{C} \) (\( \Delta \)) and leucine-UL-\( ^{14} \text{C} \) (\( \Delta \)) into the insoluble fraction, and the rate of incorporation of acetate-1-\( ^{14} \text{C} \) into the lipids (\( \times \)) of 10 potato disks after different times of aging.

**Discussion**

The results described in this paper agree fully with the conclusions of Click and Hackett (4) that the changes observed during the aging of potato tuber slices relate to the de novo synthesis of RNA and proteins, presumably resulting from derepression soon after slicing.

While the rate of evolution of \( ^{14} \text{CO}_2 \) from acetate-1-\( ^{14} \text{C} \) increases throughout the aging period (fig 8), the rates of incorporation of various labelled substrates reach their peaks in a quick succession in the following order: lysine (first peak), uridine into RNA, leucine into protein, lysine (second peak), and acetate into lipids. The maximal rate of oxygen consumption is reached some time after the peak in fatty acid synthetase is obtained (fig 9). The first peak in the incorporation of lysine has not been characterized. The main peak of lysine incorporation corresponds approximately to the peak of leucine incorporation into proteins.

The time sequence at which the peaks in the various rates of incorporation appear, allows for some speculation about the sequence of events leading to an increased rate of respiration during aging.
Immediately after slicing, one or more repressors may be removed, and the resulting derepression may lead to the synthesis of short-lived mRNAs. These mRNAs would direct the synthesis of new proteins towards 1) the lipid synthetase systems, and as a result, an increased lipid synthesis, 2) structural proteins, able to form, in combination with the lipids, membranes of the endoplasmic reticulum, ribosomes, mitochondria, and other cytoplasmic inclusions, and 3) enzymatic proteins, associated with these membranes in structures able to support an activated metabolism. Several instances of activated metabolism expressed by a large increase in respiration have been shown to include an increase in the number of ribosomes and mitochondria. Such an increase in number of cell organelles was demonstrated in the germinating castor bean (17), peanut (2), and cotton seed (25), and in wounded sweet potato root tissue (1). Lee and Chasson give some evidence for an increase in the number of mitochondria during the aging of potato slices (15).

How the physical act of slicing could lead to the postulated derepression in potato tuber tissue is not yet known. One suggestion is that a volatile repressor escapes once the tissue is sliced. Another possible factor in derepression is the physical separation of the tissue from the center of production of a repressor. In this context, various natural growth substances (indole-acetic acid, gibberellic acid, and kinetin) and natural growth inhibitors (dormin) were tested for their possible role as repressors or inducers (3, 18, 22, 24, 27). Evidence has been presented for the predominant role of gibberellic acid in the regulation of the dormancy in the potato tuber (19, 21). Derepression has been suggested as the mechanism whereby dormancy is broken in this tissue (23). Slicing might result in either stimulating the synthesis of an inducer (e.g. gibberellic acid) at the wound surface, or separating the tissue from the site of synthesis of a repressor (e.g. dormin). This aspect of our investigations has not yet yielded conclusive results.

Although it is unlikely that aging could be prevented inside the tuber by the level of a volatile respiratory intermediate through a negative feedback control of respiration (11), such a volatile intermediate could nevertheless fulfill the same role by acting as a repressor.

The results obtained with labelled precursors of protein and RNA were not influenced by contamination of the tissue by bacteria (26). Since determination of the radioactivity remaining in the medium at the end of the various assays showed that the uptake of the labelled substrates did not markedly increase upon aging, the effects observed were not due to changes in cell membrane permeability during aging. The effects of the various inhibitors were likewise shown not to impair uptake of the labelled substrates used in the assays.

The discrepancy between the results described here and those obtained by Click and Hackett (4) are only apparent in the case of protein synthesis. These authors aged the disks by exposing them to moist air. Their results are expressed as total radioactivity incorporated during aging in the presence of the labelled substrate, while our results are expressed as rates of incorporation measured by short assays after different times of aging. In the case of the synthesis of RNA, Click and Hackett report the incorporation of only 0.25 % of the uracil-2-14C initially present in the medium after 34 hours of incubation in the presence of streptomycin. This antibiotic has since been shown highly inefficient in preventing the development of bacterial populations in aging storage disks (14). Furthermore, our investigations showed that 30 % of the uracil-2-14C taken up by the tissue had been respired as 14CO2 after only 2 hours of incubation, and that 50 % of the labelled uracil initially present in the solution had been respired after 10 hours of incubation. It is therefore doubtful that the incorporation of radioactivity from uracil, as reported by Click and Hackett, reflects RNA synthesis. In our experiments uridine-2-14C was the only precursor of RNA readily incorporated into the insoluble fraction of potato disks, without a concomitant rapid breakdown to 14CO2. Orotic acid was not taken up by the tissue.

The degree of inhibitory effect of actinomycin D on the development of fatty acid synthetase varied greatly. This variation cannot be explained by experimental error, since the effect of actinomycin D on the formation of 14CO2 from acetate-1-14C was very reproducible throughout. It is therefore suggested that, since actinomycin D acts at the level of the transcription of DNA into RNA, some RNA synthesis occurs during storage, and that RNA progressively builds up, making the tissue less sensitive to interference at the transcription level. The fact that actinomycin D, in the experiment illustrated in figure 3, hardly affected the development of fatty acid synthetase, but almost completely prevented protein synthesis, is an indication that the postulated RNA must be required for the synthesis of a few enzymes essential for subsequent development at sprouting. The activity of this RNA prior to the slicing of the tissue would imply that other limiting factors maintain the dormancy.

The results of the experiments in which actidione and actinomycin D were added and withdrawn after various times of aging cannot be fully explained with what is presently known about the aging of potato tissue. Synthesis and turnover of enzymes, stability of RNA, incorporation of acetate into protein, permeability of the cell membranes to the inhibitors, and breakdown of the inhibitors inside the cells are but a few of the interacting factors involved. Reversal of actidione
inhibition as noted in figure 5 and 6 may be related to enzymic alteration of acididione by the plant cell with concomitant decrease in inhibition.

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Literature Cited


