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Dependence of Wound-induced Respiration in Potato Slices on the Time-restricted Actinomycin-sensitive Biosynthesis of Phospholipid[1]

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ABSTRACT

Actinomycin D prevents the full development in a 24-hour period of both wound respiration and cyanide resistance only when given in the first 10 to 12 hours following the cutting of potato tuber (*Solanum tuberosum* var. Russet) slices. The capacity for choline incorporation into phosphatidylcholine increases with slice aging and is inhibited by actinomycin D in the same time-restricted way. The time-restricted effectiveness of actinomycin D applies to the cutting-elicited enhanced synthesis of three critical enzymes of phosphatidylcholine synthesis, namely phosphorylcholine-glyceride transferase, phosphorylcholine-cytidyl transferase, and phosphatidylphosphatase. By contrast, actinomycin D given at any time is without effect on the measurable levels after 24 hours of a selection of glycolytic and mitochondrial respiratory enzymes. Neither succinic dehydrogenase nor cytochrome oxidase activity increases with time in aging potato slices in the presence or absence of chloramphenicol. The foregoing observations emphasize the central role of phospholipid, and ultimately membrane biosynthesis, in the development of wound-induced respiration.

The respiration of fresh potato slices, the wound respiration, is at once three to five times that of the intact tuber on a weight basis. In approximately 24 hr following cutting, the respiration rises with time another 3- to 5-fold to yield the wound-induced, or induced, respiration. Thus, the respiration of an aged slice may be 25 times that of the intact tuber. Tuber and aged slice respiration utilize carbohydrate as substrate and comprise conventional glycolysis and the tricarboxylic acid cycle. By contrast, fresh slice respiration draws upon lipid as the primary respiratory substrate and manifests little or no tricarboxylic acid cycle activity (17, 24, 28). Lipid oxidation in fresh slices is itself unusual insofar as it constitutes a noticeable measure of α oxidation (27, 28).

While the respiration of aged slices is largely cyanide-insensitive (15, 26) and that of tubers may be sharply stimulated by cyanide (37), fresh slice respiration is predominantly cyanide-sensitive. Furthermore, aged slice respiration is markedly malonate-sensitive, whereas fresh slice respiration is not (24).

The membranes of cells of fresh slices from a variety of fleshy storage organs as viewed in the electron microscope are reduced and disorganized (2, 4, 16, 41), and mitochondria isolated from certain fresh slices (*e.g.* red beet) are manifestly degraded (41). Cells from aged slices, by contrast, show well defined ER and fully formed mitochondria with well developed cristae (29, 41). When isolated mitochondria are separated centrifugally in a density gradient, mitochondria from aged slices form a sharper, *i.e.* more homogeneous, band than mitochondria from fresh slices (8). In this context, it has been discovered that more than 30% of the phospho- and galactolipid of potato slices is degraded in 15 to 30 min at 0 C following cutting with the bulk of it resynthesized in the next 24 hr (40). The foregoing observations suggest that slicing leads to immediate membrane destruction in potato slices, and raises the prospect that respiratory recovery and overshoot, *i.e.* the development of wound-induced respiration, entails the biosynthesis of membrane components, in particular, phospholipids.

Wound-induced respiration depends on a surge of RNA and protein synthesis which follows cutting (9, 35), and one may surmise that the synthesis of one or more enzymes underlies the development of wound-induced respiration. Nevertheless, there is no glycolytic or respiratory enzyme which increases with slice aging sufficiently to explain the respiratory rise (15, 20, 33). On the other hand, a marked increase is observed in the capacity for acetate incorporation into fatty acids (43), and choline incorporation into phospholipids (3, 5, 39). In this connection, labeled choline is distributed among the various cell membranes, including mitochondrial membranes (8).

The development of wound-induced respiration is fully suppressed by inhibitors of RNA and protein synthesis, respectively (9). The general and widespread effect of the inhibition of message transcription by actinomycin gives no insight into which, if any, particular biosynthetic events are directly linked to the onset and development of wound-induced respiration. Whereas actinomycin impairs RNA synthesis in aging potato slices whenever presented, as judged by the inhibition of uracil incorporation into RNA (35), it inhibits the development of induced respiration only when given in the first 8 to 10 hr (ref. 9; see Fig. 1). We have termed this phenomenon "time-restricted inhibition," and we make use of the concept of time-restricted inhibition herein by ascertaining which of the events that may be related to the initiation and development of wound-induced respiration are susceptible to inhibition by actinomycin or other selected inhibitors solely in the first 8 to 10 hr after cutting. By the foregoing criterion, phospholipid, and presumably membrane, biosynthesis have been shown to be central to wound-induced respiratory development.

MATERIALS AND METHODS

Preparation of Slices and Treatment with Inhibitors. Potato tubers (*Solanum tuberosum* var. Russet) were kindly supplied by H. Timm, Dept. of Vegetable Crops, University of California, Davis, or purchased from the local market. One-mm-thick slices,

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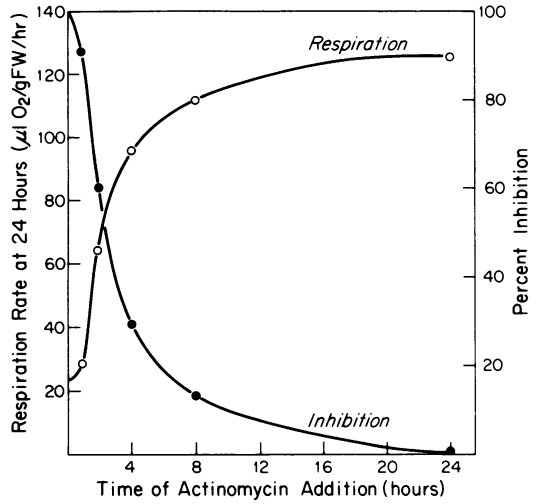

FIG. 1. Effect of actinomycin D on development of wound-induced respiration as a function of time of inhibitor presentation within a 24-hr incubation period (adapted from Click and Hackett [9]).

9 mm in diameter, were cut from the central core of the tuber, and incubated (aged) in sterile 0.1 mM calcium sulfate on a rotary shaker (23). The ratio of tissue to bathing medium was 1:10, and the solution was changed at frequent intervals to avoid contamination.

Actinomycin D (Sigma Chemical Co.) was dissolved directly in sterile, 0.1 mM calcium sulfate solution. All glassware containing solutions of actinomycin D was wrapped with aluminum foil to prevent light degradation of the antibiotic (35).

D-Chloramphenicol (Sigma Chemical Co.), 1.5 mg/ml, was added in dilute calcium sulfate solution. Erythromycin (Aldrich Chemical Co.) was first taken into a minimal volume of absolute ethanol (200 mg erythromycin/0.5 ml ethanol) or ethylene glycol monomethyl ether (200 mg erythromycin/0.1 ml ethylene glycol monoethyl ether) and then brought to a final concentration of 1 mg/ml with 0.1 mM calcium sulfate.

Application of Radioactive Lipid Precursors to Tissue Disks. For measurement of uptake and incorporation of [1,2-^{14}C]choline (21 mCi/mmol, New England Nuclear Corp.), 10 disks were incubated in 1 ml of 0.1 mM calcium sulfate solution with 0.5 μCi of labeled material.

Extraction and Isolation of Total Lipids and Estimation of Incorporated Radioactivity. After a prescribed interval in the experimental medium, disks were washed with 3 volumes of distilled H_2O to remove exogenous label, blotted with filter paper, and dropped into 5 ml of boiling isopropyl alcohol (21) for 5 min to stop enzymic activity. The isopropyl alcohol extract and the tissue residue were further blender-homogenized in 25 ml chloroform-methanol (2:1, v/v). The homogenate was passed through filter paper to remove starch and cell wall debris, and the debris washed with an additional volume of chloroform-methanol. The filtrate was then washed with 0.9% NaCl according to Folch *et al.* (13) and the phases allowed to separate overnight. The chloroform phase was removed and excess chloroform was added to the chloroform phase to remove any residual water.

Samples were taken from the chloroform phase, suspended in scintillation fluid (22), and counted in a liquid scintillation counter to determine the incorporation of [^{14}C]choline into phospholipid. An aliquot of the methanol-aqueous phase was assayed for radioactivity to determine the free [^{14}C]choline content of the tissue.

Tissue Fractionation. One hundred g of potato tissue was homogenized with an Oster vegetable juicer with 2 volumes of extraction medium consisting of 0.35 M mannitol, 0.25 M sucrose, 0.025 M TES (pH 7.8), and 0.1 mg/ml of sodium mercaptobenzothiazole (25).

The homogenate was centrifuged at 750*g* for 5 min to remove cell wall debris and starch. The 750*g* supernatant solution was then decanted into polycarbonate Oak Ridge type tubes and centrifuged in a Beckman model L ultracentrifuge at 135,000 g for 1.5 hr, utilizing a 50.1 fixed angle centrifuge head. The resulting pellet was designated the total pellet, and was resuspended in 2 ml of extraction medium. The supernatant solution was used directly in the estimation of soluble enzymes.

Mitochondria from potato were prepared as described by Laties (25).

Enzyme Assays. Choline kinase was estimated by the procedure of Tanaka *et al.* (38). After 1 hr of incubation at 25 C, the reaction was terminated by boiling the reaction medium for 3 min after which the protein precipitate was removed by centrifugation. The supernatant solution was spotted on Whatman No. 1 chromatography paper and the reaction product, phosphoryl[^{14}C]choline, was separated from radioactive choline by ascending chromatography utilizing 1-butanol-acetic acid-water (5:2:3) as the solvent system (6).

Phosphorylcholine-cytidyl transferase was assayed by the method of Moore *et al.* (32) using 0.5 μCi of [^{14}C]phosphoryl[methyl-^{14}C]choline, (40 mCi/mmol, New England Nuclear Corp.).

Phosphorylcholine-glyceride transferase was assayed utilizing a slightly modified procedure of Moore *et al.* (32). The reaction mixture contained 50 μmol of TES (pH 7.4), 10 μmol $MgCl_2$, 5 μmol 1,2-dipalmitin (presented as an emulsion in Lubrol WX), 0.1 μCi CDP[methyl-^{14}C]choline (40 mCi/mmol, New England Nuclear Corp.), and enzyme in a total volume of 0.5 ml.

The emulsion was prepared by mixing appropriate amounts of TES buffer, $MgCl_2$, and dipalmitin with 0.05% (w/v) Lubrol WX in a 10-ml plastic syringe envelope. The crude suspension was kept on ice and sonicated for three 1-min intervals with a Heat Systems Ultrasonics Sonifier model W140 with a regular tip at an output reading of 24 arbitrary units. This procedure resulted in a uniform emulsion, 0.1 ml of which was added to 0.4 ml of enzyme solution containing 0.1 μCi of CDP-[^{14}C]choline. The mixture was incubated at 30 C in a shaking water bath, and after 1 hr, the reaction was terminated with 2 ml of boiling ethanol. The protein precipitate was removed by centrifugation, and the pellet extracted with 2 ml of absolute ethanol. The ethanol extracts were then mixed with 3 ml of chloroform and partitioned twice with 5-ml aliquots of 2 M KCl. The chloroform phase was evaporated in a scintillation vial, 10 ml scintillation fluid added, and the sample assayed for radioactivity.

Phosphatidylphosphatase was assayed either by the method of Agranoff (1) utilizing lauryl phosphate (K & K Labs.) or by the method of McCaman *et al.* (31) with phosphatidic acid (Sigma Chemical Co.) as the substrate. Preparations contained 10 mg/ml BSA (fraction V, Pentex) to inhibit substrate hydrolysis by lipid acylhydrolase (14).

Glucose-6-P dehydrogenase was assayed by the method of De Moss (11) while pyruvate kinase was determined using the method of Bucher and Pfleiderer (7).

Phosphofructokinase was measured by following the rate of NADH oxidation in connection with an aldolase-α-glycerophosphate dehydrogenase couple. With 0.1 mM ATP, 1 mM fructose-6-P, 3 mM $MgCl_2$, and 0.1 M phosphate buffer (pH 7.1), the rate proved linear with total extract protein through a range of 25 to 130 μg.

Mitochondrial malate dehydrogenase was determined by following the oxidation of NADH by oxaloacetate using the reaction medium of Douce *et al.* (12). The washed mitochondrial pellet was subjected to hypotonic treatment by resuspension in 10 mM phosphate buffer (pH 7.4, without osmotic stabilizer) containing 0.5% (v/v) Triton X-100 which insured release of the matrix enzyme from the particles.

Succinate dehydrogenase was assayed by the procedure of Criddle and Schatz (10) using a Cary 14 split beam spectrophotometer. The reference cuvette contained the reaction medium without mitochondria. Absorbance of the mitochondrial suspension was balanced in the reference cuvette by appropriate screens. Cytochrome oxidase was assayed spectrophotometrically by the method of Smith (36). Protein nitrogen was determined colorimetrically with Nessler reagent following digestion.

RESULTS

Effect of Actinomycin D on Choline Incorporation into Phospholipid. Since the initial addition of actinomycin D to tissue disks has been shown to block the occurrence of induced respiration (9), we wished to determine whether the incorporation of radioactive choline into phosphatidylcholine was also curtailed by the antibiotic under the same conditions.

Actinomycin D at concentrations which have been shown to curtail the respiratory rise limited the rate of [^{14}C]choline incorporation into phosphatidylcholine. Control disks pulsed at 2-hr intervals with [^{14}C]choline during the aging period demonstrated a sigmoidal time course of radioactive choline incorporation into phospholipid. During the first 4 hr of tissue aging, incorporation was low and there was no significant difference in the rate of incorporation of labeled choline into lipid between control disks and those which were treated initially with 25 μg/ml actinomycin D (Fig. 2). However, during the 5- to 12-hr interval of aging, the rate of choline incorporation increased 5-fold in the untreated samples, while the rate of incorporation in disks treated initially with actinomycin D remained unchanged. After 12 hr, the incorporation rate in the control remained steady until termination of the experiment at 24 hr.

Choline uptake by the tissue (Fig. 3) was similar to the pattern of incorporation into phospholipid in that there was an initial 4- to 5-hr lag period after slicing followed by a subsequent 5-fold increase in the free choline concentration in the tissue in the period up to 12 hr. Absorption declined during the remainder of the 24 hr, however, to levels two to three times that of fresh tissue, while choline incorporation remained steady.

Effect of Delayed Additions of Actinomycin D on the Uptake and Incorporation of Choline during the Course of Aging. Actinomycin D inhibits the development of the induced respiration in potato disks only when added during the first 10 hr (9) (Fig. 1). Since the time of inhibitor addition is the critical factor, we examined the rate of incorporation of radioactive choline into phosphatidylcholine as affected by the time of addition of actinomycin in order to determine whether phosphatidylcholine synthesis showed time-restricted inhibition similar to that of the induced respiration.

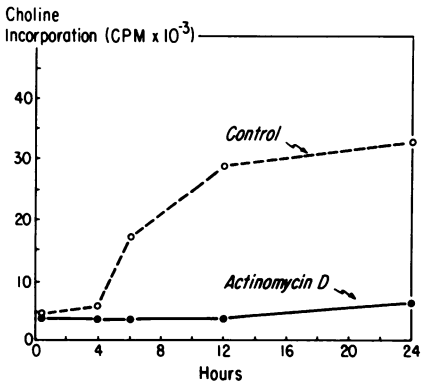

FIG. 2. Incorporation of labeled [1,2-^{14}C]choline (0.5 μCi/ml) into phosphatidylcholine in 2-hr intervals during the 24-hr course of aging. ●: disks incubated in the presence of actinomycin D (25 μg/ml) throughout the aging process.

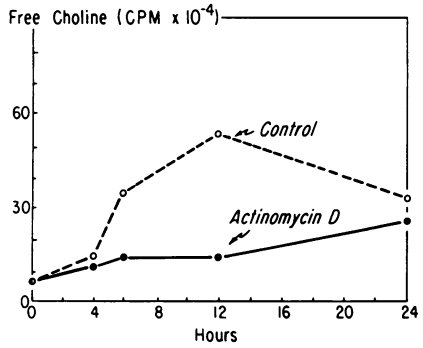

FIG. 3. Amount of free labeled choline in tissue after 2-hr absorption periods in medium containing radioactive [1,2-^{14}C]choline (0.5 μCi/ml). ●: free choline in disks aged in the presence of actinomycin D (25 μg/ml).

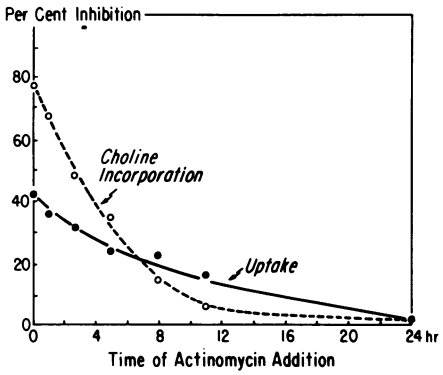

FIG. 4. Effect of delayed additions of actinomycin D (25 μg/ml) on uptake and incorporation of [1,2-^{14}C]choline (0.5 μCi/ml) into phosphatidylcholine. Inhibitor was added after 0, 1, 2.5, 5, 8, or 11 hr of incubation. Rate of uptake and incorporation was determined in a 2-hr interval at the end of 24 hr.

The addition of actinomycin D to fresh slices at the outset had the greatest effect on the subsequent incorporation of labeled choline into phosphatidylcholine. Later additions had a diminishing inhibitory effect (Fig. 4). After 10 hr of tissue aging, addition of actinomycin had no effect on the subsequent incorporation of labeled choline into lecithin.

While the inhibition of choline uptake as a function of the time of actinomycin addition was not strictly negatively linear, there was little evidence of a pronounced critical initial 10-hr period of inhibitor sensitivity as for choline incorporation (Fig. 4).

Activity of Phosphatidylcholine Biosynthetic Enzymes Compared with Key Glycolytic and Respiratory Enzymes during the Course of Aging. The susceptibility to actinomycin of *in vivo* choline incorporation into phosphatidylcholine during the initial 10-hr critical period of aging raised the question of whether increased phospholipid synthesis with aging was due to an increase in synthesis of cellular enzymes in general, or reflected in part a specific rise of the enzymes implementing phosphatidylcholine biosynthesis. To answer this question, key enzymes of the lecithin biosynthetic path, and of the glycolytic, pentose-phosphate, and mitochondrial respiratory pathways were assayed in homogenates of tissue disks of fresh and aged slices. Time-restricted biosynthesis of enzymes was differentiated from time-indifferent biosynthesis of enzymes by presentation of actinomycin D to tissue disks at intervals during aging, followed by determination of the total enzyme activity at the end of the 24 hr.

In both fresh and aged potato disks, phosphorylcholine-glyceride transferase (EC 2.7.8.2) was found to be associated solely with the membranous fraction sedimented at 135,000*g* for 1.5

Table I. The Effect of Aging on Enzymatic Activity in Potato Disks

Enzymatic activity was assayed as described in the Materials and Methods section. The relative activity on a protein basis is expressed as the activity observed in a 24 hour aged sample divided by the activity found in the fresh sample.

Enzyme	Number of Experiments	Relative Activity (aged/fresh)
Phosphorylcholine-glyceride transferase	5	2.79 ± 0.90
Phosphorylcholine-cytidyl transferase	1	1.96 ± 0.00
Phosphatidylphosphatase	2	1.93 ± 0.20
Choline kinase	2	1.32 ± 0.28
Pyruvate kinase	2	1.50 ± 0.35
Phosphofructokinase	2	1.40 ± 0.09
Glucose 6-phosphate dehydrogenase	2	1.28 ± 0.20
Mitochondrial malate dehydrogenase	2	1.15 ± 0.32
Cytochrome c oxidase	2	0.89 ± 0.08

hr. The total activity of the membrane-bound enzyme increased 2- to 3-fold during the course of aging (Table I). Phosphorylcholine-cytidyl transferase (EC 2.7.7.15) was also found primarily in the pellet, although a small fraction (10%) of the enzyme was consistently associated with the supernatant fraction in both fresh and aged tissue. When the total activity of pellets from fresh and aged tissue is compared, there is about a 2-fold increase with age. Phosphatidylphosphatase increased almost twice (Table I). The enzyme was associated with the high speed sedimentable membranous pellet. Choline kinase (EC 2.7.1.32), which yields phosphorylcholine from choline and ATP, was associated with the supernatant fraction and increased some 20% with tissue aging.

When aging disks were exposed to actinomycin D at different times during the aging process, the activity of phosphorylcholine-glyceride transferase after 24 hr of aging was found to be severely limited only when actinomycin was added during the first 10 hr (Fig. 5). Phosphatidylphosphatase showed a similar pattern of sensitivity to the time of addition of actinomycin D. To a lesser degree, sensitivity to actinomycin D of the development of phosphorylcholine-cytidyl transferase activity was also limited to the first 10 hr after slicing.

In sharp contrast to these enzymes, choline kinase (EC 2.7.1.32) showed an almost linear negative relation of inhibition with time of actinomycin addition, and inhibition was at a low level at best.

When the effect of the time of addition of actinomycin D during the aging process was examined with respect to the enzymes pyruvate kinase, P-fructokinase, glucose-6-P dehydrogenase, and mitochondrial malate dehydrogenase, there was a completely different response from that observed for phosphorylcholine-glyceride transferase, phosphatidylphosphatase, and phosphorylcholine-cytidyl transferase (Fig. 6). Glucose-6-P dehydrogenase and mitochondrial malate dehydrogenase showed no sensitivity to actinomycin D presented at any period of aging. Pyruvate kinase showed a low level of inhibition of activity for the first 2 hr. Inhibition declined with subsequent addition of actinomycin D, until there was no effect at all after 5 hr. Phosphofructokinase activity was unaffected initially and very little affected by actinomycin D addition in the first 5 hr (Fig. 6).

Effect of Chloramphenicol and Erythromycin on the Development of Induced Respiration. The possible contribution of mitochondrial protein synthesis to the attainment of induced respiration was assessed in the presence of either chloramphenicol or erythromycin, both known inhibitors of mitochondrial protein synthesis (30, 34). In the presence of chloramphenicol, there was no essential difference in the pattern of induced respiration when compared with control aged disks. Erythromycin, an alternative inhibitor of mitochondrial protein synthesis, also had no effect on induced respiration. The development of cyanide insensitivity, which is characteristic of aged tissue slices, was also observed in chloramphenicol-aged material.

Activity of Mitochondrial Enzymes in Slices Aged in Chloramphenicol. The activity of several mitochondrial enzymes was compared from fresh, 24-hr aged, and 24-hr chloramphenicol-aged tissue in order to determine if there was augmented synthesis or turnover of these enzymes during the course of wound-induced respiration.

Mitochondria from aged and chloramphenicol-aged tissue had similar Cyt *c* oxidase activity which was less than that of fresh tissue on both a total and a specific activity basis (Table II). Succinic dehydrogenase activity was also greater in fresh tissue

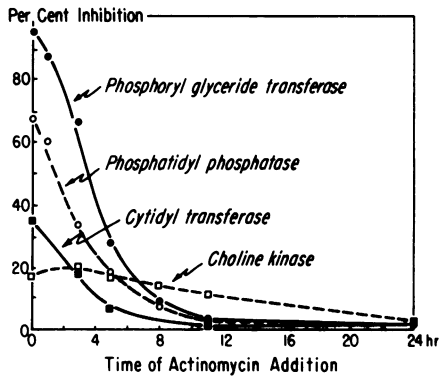

FIG. 5. Effect of time of addition of actinomycin D (25 μg/ml) on enzymic activities of phosphorylcholine-glyceride transferase, phosphatidylphosphatase, phosphorylcholine-cytidyl transferase, and choline kinase. Inhibitor was added after 0, 1, 2.5, 5, 8, or 11 hr of disk incubation and the enzymic activity of the supernatant fraction and total pellet measured at the end of 24 hr of disk aging in dilute calcium sulfate solution.

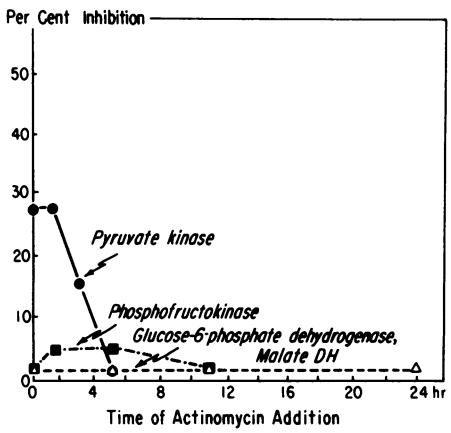

FIG. 6. Effect of delayed additions of actinomycin D (25 μg/ml) on pyruvate kinase, phosphofructokinase, mitochondrial malate dehydrogenase, and glucose-6-P dehydrogenase.

Table II. The Activity of Mitochondrial Inner Membrane and Matrix Enzymes in Fresh, 24 Hour Aged, and 24 Hour Chloramphenicol Aged Tissue Slices

Enzymatic activity was assayed as described in the Materials and Methods section. The change in enzyme activity is expressed on a relative basis as the activity observed in the 24 hour aged sample divided by the activity found in the fresh sample.

Enzyme and Treatment	Relative Activity (aged/fresh)
Control	
Cytochrome c oxidase	0.88
Malate dehydrogenase	0.79
Succinic dehydrogenase	0.81
Chloramphenicol (1.5 mg/ml)	
Cytochrome c oxidase	0.90
Malate dehydrogenase	0.83
Succinate dehydrogenase	0.84

than in aged and antibiotic-incubated samples, wherein activity was similar (Table II). The mitochondrial matrix enzyme, malate dehydrogenase, followed the same pattern of high total and specific activity associated with fresh tissue, and lowered activities in the aged material. Chloramphenicol in the aging solution did not affect the level of malate dehydrogenase in mitochondria from aged disks compared with untreated controls.

DISCUSSION

While the inhibition of respiratory development in slices of bulky storage organs by actinomycin points to the role of RNA synthesis and attendant protein synthesis in the development process, there is no indication of which of the many possible biosynthetic events are causative and which are consequential in the initiation and course of wound-induced respiration. In this connection, it is of paramount importance that the inhibitory effect of actinomycin on the full course of respiratory development through 24 hr is evident only when actinomycin is given in the first 8 to 10 hr after slicing (9, 35). Seemingly, the one or more messages which control the development of induced respiration are transcribed soon after cutting and translated in the course of the next 24 hr. While a variety of glycolytic enzymes increase with aging of potato slices (20) and their turnover and *de novo* synthesis have been demonstrated (19), evidence of induced transcription in response to cutting is lacking, and there is no indication that they are causatively implicated in the development of the induced respiration in the critical first 8 to 10 hr.

In our experiments, actinomycin is presented at varying intervals from the time of cutting, the metabolic measurement (*e.g.* choline incorporation, enzyme activity) being made in a 2-hr period at the end of 24 hr. This way there need be but one control, and an integrated value is obtained through several hr of the effect of the experimental variable. That is, the readout is multiplied so to speak, a condition which is a virtue so long as the turnover time of the biosynthetic product is not too short in relation to the total experimental period. That such is the case is manifest in the absence of inhibitor effect when inhibitor is given after 10 hr.

In our experiments the concept of time-restricted inhibition was put to use to determine which actinomycin-sensitive events of potentially many are central to the evocation and development of induced respiration. Since glycolysis, pentosephosphate cycle, and tricarboxylic acid cycle activity are sharply enhanced in connection with wound-induced respiration, it might have been anticipated that one or another of the enzymes comprising the foregoing sequences would show an actinomycin-sensitive rise. Such was not the case. Rather, three enzymes involved in lecithin biosynthesis—*viz.* phosphorylcholine-cytidyl transferase, phosphorylcholine-glyceride transferase, and phosphatidylphosphatase fulfilled the specified criteria. While the rise in activity of these enzymes was modest, *i.e.* from two to three times, the rise was actinomycin-sensitive in a time-restricted way. Evidence suggests that the message for at least one of these enzymes, phosphorylcholine-glyceride transferase, is labile, since disks treated with actinomycin immediately on cutting show a 96% diminution of enzyme activity after a day compared with the 24-hr control. That is, there is an absolute drop in enzyme level in the presence of actinomycin, signifying an actinomycin-sensitive turnover. It is of interest that enzymes which develop in barley aleurone in response to gibberellin (18) and in castor bean seeds on germination (32) are included in this group.

The course of choline incorporation follows the same actinomycin-sensitive time-restricted pattern as do the three lecithin biosynthetic enzymes. Choline uptake *per se* is less sensitive to actinomycin and is not sharply time-restricted. As will be shown in a following paper (42), a choline analogue, dimethylaminoethanol, inhibits the development of induced respiration in a time-restricted way, as does cerulenin, an inhibitor of fatty acid biosynthesis. The presumption stemming from our observations is that phospholipid biosynthesis underlies membrane biosynthesis, which in turn controls the development of induced respiration. Since mitochondrial activity increases while mitochondrial numbers do not—as judged by the absence of any increase in Cyt oxidase, and the absence of any effect of erythromycin or chloramphenicol on the level of Cyt oxidase—we are led to the view that intussusception of new mitochondrial membrane components accounts for the ultimate enhancement of mitochondrial activity in aged slices over that in the intact tuber. In this connection mitochondrial phospholipids are degraded to an unmeasurable extent during mitochondrial isolation (39) and it is for this reason that it is difficult to make a significant quantitative comparison of the phospholipid composition of mitochondria from fresh and aged tissue. Nevertheless, we have noted that the per cent of the total mitochondrial fatty acid comprised by oleic acid, for example, changes from 2.5 to 7% with aging (Theologis and Laties, unpublished) and it is this type of observation together with earlier isotopic evidence of phospholipid synthesis in mitochondria from aged slices (8) which causes us to support the foregoing hypothesis.

A simple quantitative comparison of the respiratory activity of aged and fresh slices as a measure of relative mitochondrial activity is ruled out because of the anomalous nature of fresh slice respiration (17, 27). Perhaps the very substrates for fresh slice respiration—the free fatty acids arising from membrane lipid breakdown—may prove to be derepressors in aging disks which evoke membrane biosynthesis and its respiratory consequences.

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