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Membrane Transformations in Aging Potato Tuber Slices[1]

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ABSTRACT

When potato tuber slices (*Solanum tuberosum* L.) are incubated with radioactive choline, labeled membrane-bound phospholipids are formed. If potato slices are aged for 0 to 24 hours before exposure to radioactive choline, the distribution of the labeled phospholipids undergoes both quantitative and qualitative changes. Quantitatively, there is a marked increase in the total lipoidal radioactivity with aging time. Qualitatively, there is a shift in the kinds of subcellular fractions that are being labeled. Fresh slices incorporate most of the lipoidal radioactivity in the microsomes. Slices aged for 9 hours incorporate most of the label in a fraction consisting of single membrane-bound cisternae, which are presumed to be dictyosomal fragments. Slices aged for 24 hours before incubation with radioactive choline incorporate the greater portion of the label in this same fraction, but a significant portion of the label is found in a heavier, mitochondria-containing fraction.

When thin slices are prepared from bulky storage tissues and incubated aerobically, there is a marked increase in respiration. This phenomenon was observed in potato tuber as early as 1887 by Boehm (7). The most interesting point about this phenomenon is that the respiratory increment is qualitatively distinguishable from the initial or basal respiration. Throughout the years, numerous hypotheses have been advanced to explain the induction of these respiratory changes by slicing. One of the most fruitful has been the derepression hypothesis, first suggested by Click and Hackett (8), according to which this respiratory rise is dependent upon specific synthesis of RNA and protein. The later observations by Willemot and Stumpf (26), Leaver and Key (14), and others support the derepression hypothesis.

Evidence for mitochondrial biogenesis in aging root and tuber slices has been presented by several workers (1, 2, 6, 15, 23, 25). In all these cases, tissue slices were aged for at least 24 hr before a mitochondrial increase was demonstrated.

The incorporation of radioactive precursors into the phospholipids of aging potato tuber slices was investigated by Tang and Castelfranco (22), Ben Abdelkader (4), and Ben Abdelkader and Mazliak (5).

In the present study we have undertaken to separate the membranous cell constituents after labeling potato slices with radioactive choline. Our results indicate that during aging there is a qualitative shift in the kinds of membranes that are synthesized from exogenous choline. In the fresh slices the label is incorporated into the lipids of a particularly "light" membrane fraction. After 9 hr of aging, most of the lipid-soluble radioactivity occurs in an "intermediate" band, while, after 24 hr, a considerable portion of the label is found in a "heavy" band which overlaps succinoxidase activity on the gradient.

MATERIALS AND METHODS

The source of materials and the basic procedures for preparation of potato tuber slices, aging, the incorporation of radioactive lipid precursors into slices, and the assay of lipid-soluble radioactivity have been described previously (22).

Cell Fractionation Procedures. Several cell fractionation procedures were employed. The first was a slight modification of the method of Verleur (24). Fifteen grams of slices were ground with 60 ml of ice-cold medium of the following composition: 0.7 M mannitol; 10 mM potassium phosphate, pH 6.5; 0.1% BSA[4] fraction V, fatty acid poor; 1 mM EDTA; and 2 mM cysteine-HCl. These were ground for 20 sec at 50 v in a VirTis homogenizer. After passing through two layers of cheesecloth, the homogenate was centrifuged at 100*g* for 10 min in a Servall refrigerated centrifuge, and the precipitate was discarded. The supernatant was then centrifuged at 10,000*g* for 10 min to spin down the mitochondria. The pellet was washed once with a buffer system containing 0.7 M mannitol, 10 mM phosphate (pH 6.5), and 0.1% BSA and centrifuged, and the resulting pellet was called "crude mitochondria." Microsomes were obtained by centrifugation of the mitochondrial supernatant at 105,000*g* for 90 min in a Spinco No. 40 rotor. The supernatant thus obtained was called "final supernatant."

An alternate cell fractionation procedure was more suitable for small samples of material and appeared to be less destructive to the isolated organelles. In a typical experiment, 3.5 g of potato slices were hand-ground with a mortar and pestle without abrasive in ice-cold buffer of the following composition: 0.5 M mannitol; 10 mM potassium phosphate, pH 6.5; 2 mM cysteine-HCl; 2 mM $MgCl_2$, and 1 mM EDTA. The homogenate was adjusted to 25-ml volume and subjected to the following centrifugation schedule: (*a*) centrifuge homogenate, 5 min at 500*g*: pellet, P_1, and supernatant, S_1; (*b*) centrifuge S_1, 15 min at 20,000*g*: pellet, P_2, and supernatant, S_2; (*c*) centrifuge S_2, 60 min at 105,000*g*: pellet, P_3, and supernatant, S.

Samples to be fractionated by density gradient centrifugation were homogenized by the following procedure. One hundred potato slices weighing approximately 4.5 g were ground in a mortar and pestle at 0 C in a buffer of the following composition: 20% sucrose (w/w); 0.1% BSA; 10 mM potassium phosphate buffer, pH 6.5; 1 mM $CaCl_2$; and 1 mM cysteine-

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[4] Abbreviation; BSA: bovine serum albumin.

HCl. The homogenate was adjusted to a 10-ml volume. Nine milliliters of this slurry were applied on top of a 24-ml linear density gradient from 30 to 55% sucrose (w/w) contained in a cellulose nitrate cup. The gradient also contained: 10 mM potassium phosphate buffer (pH 6.5), 1 mM cysteine-HCl, and 1 mM $CaCl_2$.

The gradients were centrifuged for 30 min at 24,500 rpm in an SW-25 Beckman Spinco rotor (average gravitational field about 50,000*g*). The gradients were cut rapidly (3.0 ml/min) into 1.5-ml fractions, starting from the top, with an Isco gradient fractionator model 180. The operation was carried out in a 4 C room.

Sodium cacodylate was substituted in the above procedure for potassium phosphate buffer at the same pH and concentration in both grinding medium and gradient whenever the fractions were to be assayed for glucose 6-phosphatase activity.

Gradient fractions to be extracted for the determination of the total lipid-soluble radioactivity were first treated with one-tenth of their volume of 1 M NaF and boiled for 5 min to inactivate the lecithin-degrading enzymes.

The incorporation of leucine-U-^{14}C into proteins of subcellular organelles was assayed by adding an equal volume of 10% trichloroacetic acid containing 0.1 M leucine-^{12}C to each sample. After 30 min at 4 C, the proteins were collected by centrifugation and washed once again with 5% trichloroacetic acid containing 50 mM leucine-^{12}C. The precipitate was then transferred to a planchet and counted in a gas flow counter. Whenever necessary, BSA was added as a carrier.

Succinoxidase. Succinoxidase activity was determined in the gradient fractions with an oxygen electrode (Yellow Springs Instrument Co., Inc., model 53 Biological Oxygen Monitor) at 25 C. To the reaction mixture were added BSA, 3 mg; potassium phosphate buffer, pH 7.3, 60 μmoles; $MgCl_2$, 15 μmoles; EDTA, 3 μmoles; cytochrome *c*, 15 nmoles; ADP, 1 μmole; gradient fraction, 1.0 ml; and sodium succinate, 30 μmoles, in a total volume of 3.0 ml. A base line was established before the reaction was started by the addition of sodium succinate. When the reaction had run for a while, 150 μmoles of sodium malonate were added. "Succinoxidase activity" was defined as the rate of O_2 uptake after sodium succinate addition minus the rate of O_2 uptake after sodium malonate addition and expressed as nmoles O_2 taken up/min·3 ml reaction chamber.

Table I. *Distribution of Lipoidal or Proteinaceous Radioactivity between the Crude Mitochondrial and Microsomal Fractions Isolated from Potato Slices Labeled with Various Precursors*

Fifteen grams of either fresh or 9-hr-old slices were incubated in 50 ml of $CaSO_4$-chloramphenicol solution containing 5 μc of radioactive precursor (choline-1,2-^{14}C, acetate-1-^{14}C, or leucine-U-^{14}C) for 2 hr. Mitochondrial and microsomal pellets were isolated according to Verleur (24).

	Constituents Analyzed	Radioactivity			
		Mitochondria		Microsomes	
		cpm	*aged/fresh*	*cpm*	*aged/fresh*
Acetate-1-^{14}C Fresh	Lipids	30,000	1.80	77,300	1.68
9-hr-old		54,000		129,400	
Choline-1,2-^{14}C Fresh	Lipids	2,000	28.0	8,000	17.7
9-hr-old		56,000		141,600	
Leucine-U-^{14}C Fresh	Proteins	550	47.9	4,440	28.2
9-hr old		26,350		125,000	

Whole Slice Respiration. The rate of O_2 uptake by whole slices was measured with the O_2 electrode. The assay system contained 30 slices (about 1.4 g tissue) in 4.0 ml of 0.1 M potassium phosphate, pH 7.0, at 25 C.

Glucose 6-Phosphatase. Glucose 6-phosphatase activity was determined by a procedure adapted from Nordlie and Arion (19). To each reaction mixture were added sodium cacodylate buffer, pH 6.5, 45 μmoles; sodium glucose-6-P, 15 μmoles; gradient band, 0.5 ml in a total volume of 0.75 ml. The tubes were incubated at 28 C for 1 hr, and the reaction was stopped by adding 0.5 ml of 10% trichloroacetic acid and chilling immediately. A zero time control was run for each fraction. After centrifugation, 1 ml of the supernatant was assayed for inorganic phosphate by the Fiske-SubbaRow method, using the bisulfite-aminonaphthol sulfonic acid reducing agent prepared according to Flynn *et al.* (11). "Glucose 6-phosphatase activity" was defined as the μmoles of P_i liberated during the incubation.

Preparation of Lecithin Micelles. Lecithin micelles were prepared according to Attwood and Saunders (3) and Dallner *et al.* (9); 0.25 g of beef lecithin was dissolved in ether and 15 ml of buffer (0.7 M mannitol and 10 mM potassium phosphate, pH 6.5) were added. Lecithin sol was prepared by driving the ether off under reduced pressure. The volume of the sol was then adjusted to 15 ml with the same buffer. Lecithin micelles were obtained by ultrasonication of the sol three successive times, each for 5 min at 60 w using a Biosonik II apparatus (0.5-inch titanium tip). An ice bath was used to control the temperature.

RESULTS

Slices incubated with radioactive lipid or protein precursors were fractionated using the modified procedure of Verleur (24) described in "Materials and Methods." The radioactivity present in the total lipids or in the trichloroacetic-insoluble proteins was determined for each fraction.

A comparison of the radioactivity found in crude mitochondria and microsomes of both fresh and 9-hr-old slices is shown in Table I. In the case of fresh slices, the amount of labeled choline and leucine incorporated into lipids and protein, respectively, was very slight. There was a large increase in both the lipoidal and proteinaceous radioactivity after 9 hr of aging. In this study the microsomal fraction possessed most of the radioactivity, regardless of which precursor was used. When labeled acetate served as the precursor, the trend was qualitatively the same, except that a much higher label incorporation was observed in the fresh slices from ^{14}C-acetate than from the other radioactive precursors.

However, if the potato slices were ground in a mortar and pestle without abrasive and subjected to the differential centrifugation scheme according to the alternate procedure described in "Materials and Methods," most of the radioactivity incorporated into lipids from choline-1,2-^{14}C was found in the "crude mitochondrial fraction," which was spun down at 15 min, 20,000*g*, but not at 5 min, 500*g* (Table III).

It appears that in our modification of Verleur's isolation procedure (24) the mechanical homogenizer is responsible for disrupting a fragile subcellular particle, the fragments of which are brought down in the microsomal pellet. The recovery of the lipid-soluble radioactivity from ^{14}C-choline is low (Tables II and III) in comparison with recovery of radioactivity from ^{14}C-acetate and ^{14}C-leucine, which approaches 100% (Table II). The possibility that the radioactive phospholipid, synthesized *in vivo* from exogenous radiocholine, might in fact be free lecithin adsorbed by various cell fragments but not an integral part of any specific membrane system

Table II. *Distribution of Lipoidal or Proteinaceous Radioactivity in Potato Slices Incubated with Acetate-1-^{14}C, Choline-1,2-^{14}C, or Leucine-U-^{14}C*

Fifteen grams of 9-hr-old potato slices were incubated in 50 ml of $CaSO_4$-chloramphenicol solution containing 5 μc of either acetate-1-^{14}C, choline-1,2-^{14}C, or leucine-U-^{14}C for 2 hr. The tissue was fractionated according to a procedure adopted from Verleur (24). The total radioactivity before fractionation was determined on identical tissue samples incubated in the same way. These samples were extracted directly with chloroform-methanol (22) for the determination of lipoidal radioactivity and with dilute trichloroacetic acid for the determination of proteinaceous radioactivity. The total lipoidal radioactivity derived from acetate-1-^{14}C was 250,000 cpm, and that from choline-1,2-^{14}C, 75,000 cpm; the proteinaceous radioactivity was 265,000 cpm.

Fraction	Proteinaceous Radioactivity	Lipoidal Radioactivity	
	Leucine-U-^{14}C	Acetate-^{14}C	Choline-1,2-^{14}C
	cpm		
Cheesecloth		4,300	4,000
Pellet 100*g*		3,800	1,000
Crude mitochondria	26,350	54,700	12,000
Microsomes	125,000	129,400	29,000
Final supernatant	87,100	84,000	2,600
Total radioactivity recovered	238,450	276,200	48,600

was tested by repeated washing of the radioactive microsomal pellet with lecithin micelles.

Fifteen grams of 9-hr-old potato slices were incubated in 50 ml of $CaSO_4$-chloramphenicol solution containing 50 μc of ^{3}H-choline for 2 hr and fractionated according to the procedure adapted from Verleur (24). Microsomes were isolated. The microsomal pellet corresponding to 2 g of potato slices was suspended in 2 ml of lecithin micelles and recentrifuged at 105,000*g* for 30 min. The pellet obtained was washed two more times; the chloroform-soluble radioactivity in the final pellet and in the three washes was counted. In duplicate experiments, 13 to 16% of the recovered radioactivity was found in the first wash, 8 to 10% in the second wash, 5 to 7% in the third wash, and 68 to 74% in the pellet.

Since chloramphenicol has been shown to inhibit protein synthesis, not only in bacteria but also in plant mitochondria (10), it was decided to avoid the use of antibiotics. In the subsequent work on membrane separation, all the materials used in the preparation of potato slices were autoclaved; the potato skin was surface-sterilized with 70% alcohol. The manipulations were performed in a sterile transfer room.

The first attempts to characterize the membranes containing the radioactive phospholipids failed because of the speed with which the radioactive lecithin breaks down as these membranes are handled. Later, the procedure described in "Materials and Methods" for the gradient separation of radioactive membranes was developed, which permitted us to examine these membranes while recovering 60 to 80% of the original label. In this procedure the starch and cell wall debris are not removed before the brei is applied on the top of the gradient. These are spun down to the bottom of the centrifuge tube with about 6% of the lipid-soluble radioactivity. The profile obtained with potato slices aged for 9 hr and incubated for 2 hr with choline 1,2-^{14}C shows a double peak near the interface between the sample zone and the gradient and a smear of radioactivity in the gradient itself (Fig. 1). A similar gradient was

Table III. *Distribution of Radioactive Lipids Formed by Incubating Potato Slices with Choline-1,2-^{14}C*

Twenty potato slices (weighing about 0.9 g) were aged for 2 or 9 hr in 0.1 mM $CaSO_4$ and incubated for 2 hr in 2 ml of 0.1 mM $CaSO_4$ containing 1 μc of choline-1,2-^{14}C. The tissue was hand-ground in a mortar and pestle without abrasive and fractionated as reported in "Materials and Methods." The total radioactivity before fractionation was determined on an equal number of slices contained in another flask and incubated in the same way. At the end of the incubation these slices were rinsed, blotted, and extracted directly with chloroform-methanol (22). On this basis, we estimate the total recovery of lipoidal radioactivity to be 79% for the 2-hr-old slices and 88% for the 9-hr-old slices.

	Lipid-soluble Radioactivity	
	Aged 2 hr	Aged 9 hr
	cpm	
Total radioactivity before fractionation	41,600	66,700
P_1 debris	5,880	10,700
P_2' "crude mitochondria"	21,100	36,300
P_3' "microsomes"	4,750	6,500
S, supernatant	1,150	4,900
Total	32,880	58,400

centrifuged for an additional 5 hr (*i.e.*, for a total of 5.5 hr), while an aliquot of the original homogenate was stored at 4 C; this aliquot suffered a 38% loss of its radioactive lipids during this interval. The long centrifugation caused the lighter peak to move slightly into the gradient, with a maximum corresponding to a buoyant density of approximately 1.115 g/cc (Fig. 2, band A). The radioactivity which was previously smeared throughout the gradient formed a broad band with a maximum at the buoyant density of 1.187 g/cc (Fig. 2, band B). Recovery of the lipid-soluble radioactivity from the gradient fraction was 52.6% of the label in the sample.

The equilibrium-buoyant density of approximately 1.187 g/cc for the radioactive peak suggests a mitochondrial band. Particles possessing succinoxidase activity do in fact band in this gradient system (Figs. 3 and 6), reaching in 30 min a posi-

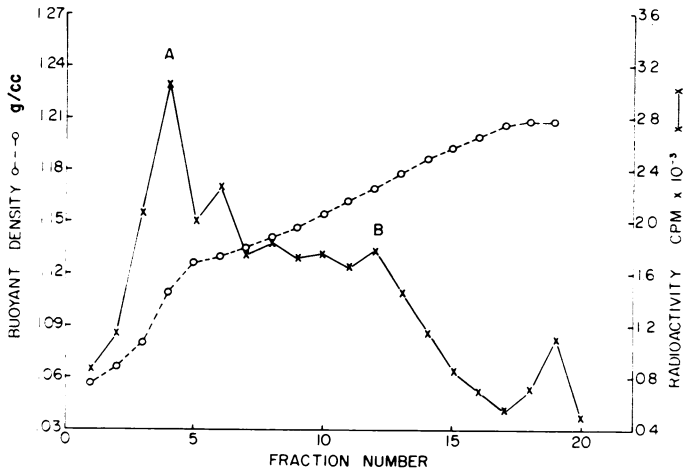

FIG. 1. Distribution of the lipoidal radioactivity on the sucrose density gradient after 30 min centrifugation at 50,000*g*. Potato slices were aged for 9 hr, then incubated with choline-1,2-^{14}C for 2 hr. A and B indicate the positions of the two major bands. Potassium phosphate buffer was used.

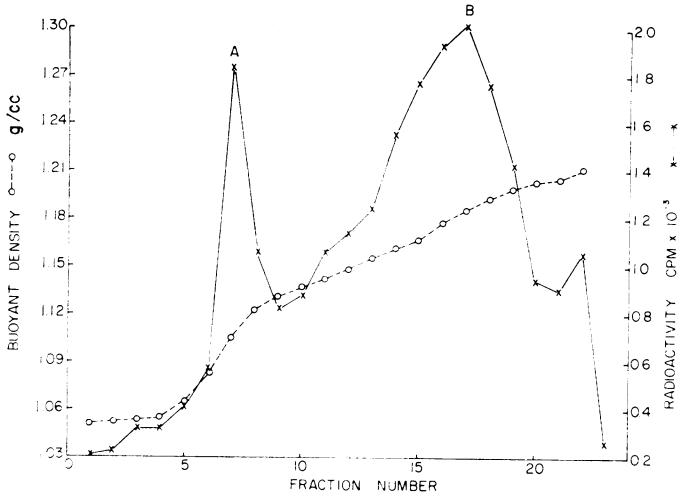

FIG. 2. Distribution of the lipoidal radioactivity on the sucrose density gradient after 5.5 hr centrifugation at 50,000*g*. Potato slices were aged for 9 hr, then incubated with choline-1,2-^{14}C for 2 hr. A and B indicate the positions of the two major bands. Potassium phosphate buffer was used.

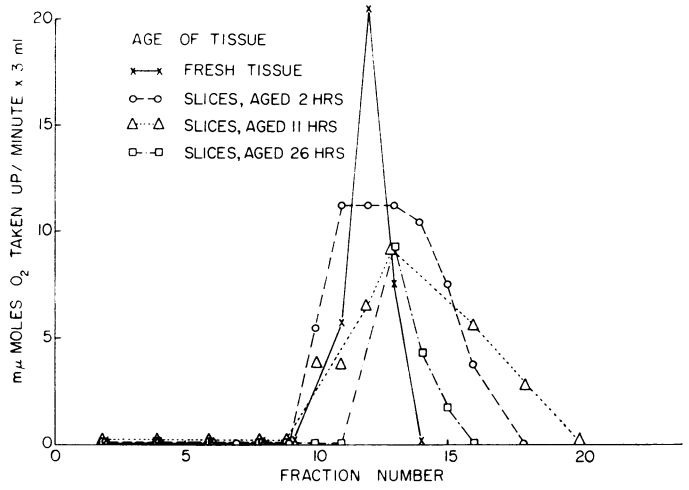

FIG. 3. Distribution of succinoxidase activity on the sucrose density gradient after 30 min centrifugation at 50,000*g*. Sodium cacodylate buffer was used.

tion which corresponds to the peak marked "B" in Figure 1. Slicing and subsequent aging cause marked changes in the behavior of the succinoxidase band (Fig. 3). Mitochondria extracted from fresh potato tissue give a sharp band; mitochondria extracted from slices aged for 2, 11, or 26 hr in 0.1 mM $CaSO_4$ give more or less diffuse bands (Figs. 3 and 6). These changes appear to be caused directly or indirectly by the mechanical injury during slicing (23).

The density gradient profiles of lipid-soluble radioactivity were compared with the succinoxidase and glucose 6-phosphatase profiles. The experiments summarized in Figures 3, 4, and 5 were run under the same conditions but not simultaneously. In the grinding and the density gradients, 10 mM sodium cacodylate, pH 6.5, was substituted for the 10 mM potassium phosphate normally used. Cacodylate has no adverse effect upon either choline-1,2-^{14}C incorporation into phospholipids or succinoxidase; this substitution was made necessary by the glucose 6-phosphatase assay (11).

The glucose 6-phosphatase profiles corresponding to different tissue ages (Fig. 4) indicate that the level of this enzymatic activity decreases during the first 11 hr of aging. Qualitatively, these changes parallel what happens to the succinoxidase activity (Fig. 3) and can be interpreted also as the result of mechanical injury to the endoplasmic reticulum (13). Practically all the glucose 6-phosphatase activity is found in tubes 1 through 6, indicating that under our centrifugation conditions the glucose 6-phosphatase-containing microsomes (12) do not pass out of the sample zone into the gradient.

The profiles of the lipid-soluble radioactivity show some striking changes with aging. Most striking is the variation in total amount of label found in the chloroform extract. After 9 hr of aging, the tissue incorporated 6 times as much radioactivity, and, after 24 hr of aging, 26 times as much radioactivity as the fresh control (Fig. 5). In the fresh control, the bulk of the lipid-soluble radioactivity was found in tubes 1 to 6, *i.e.*, in the sample zone. In the 9-hr-activated tissue, a radioactive peak appeared at the transition between sample zone and gradient. This peak was followed by a smear of radioactivity down to tube 16.

In the 24-hr-activated tissue, the main peak was still near the transition zone. However, a second large peak had appeared

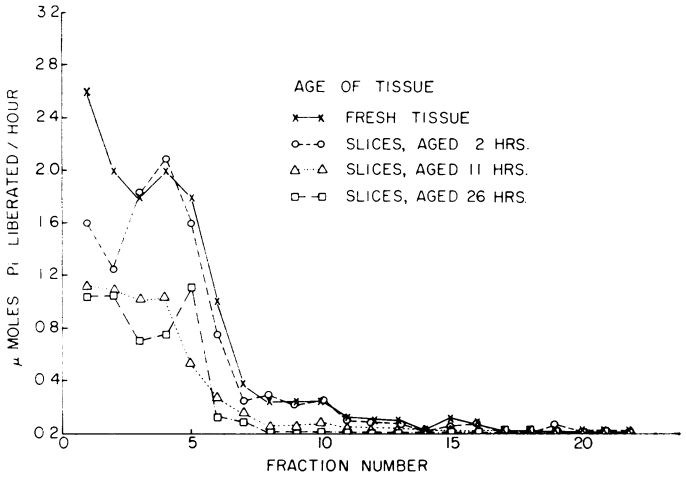

FIG. 4. Distribution of glucose-6-phosphatase activity on the sucrose density gradient after 30 min centrifugation at 50,000*g*. Sodium cacodylate buffer was used.

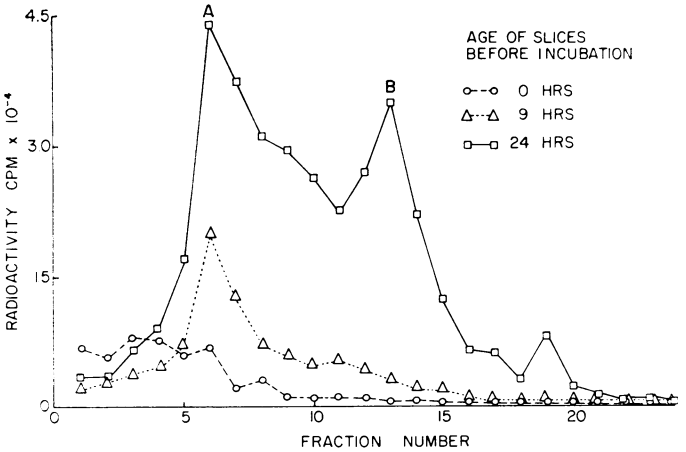

FIG. 5. Distribution of the lipoidal radioactivity on the sucrose density gradient after 30 min centrifugation at 50,000*g*. Potato slices were aged for different periods before the 2 hr incubation with choline-1,2-^{14}C. A and B indicate the positions of the two major bands. Sodium cacodylate buffer was used.

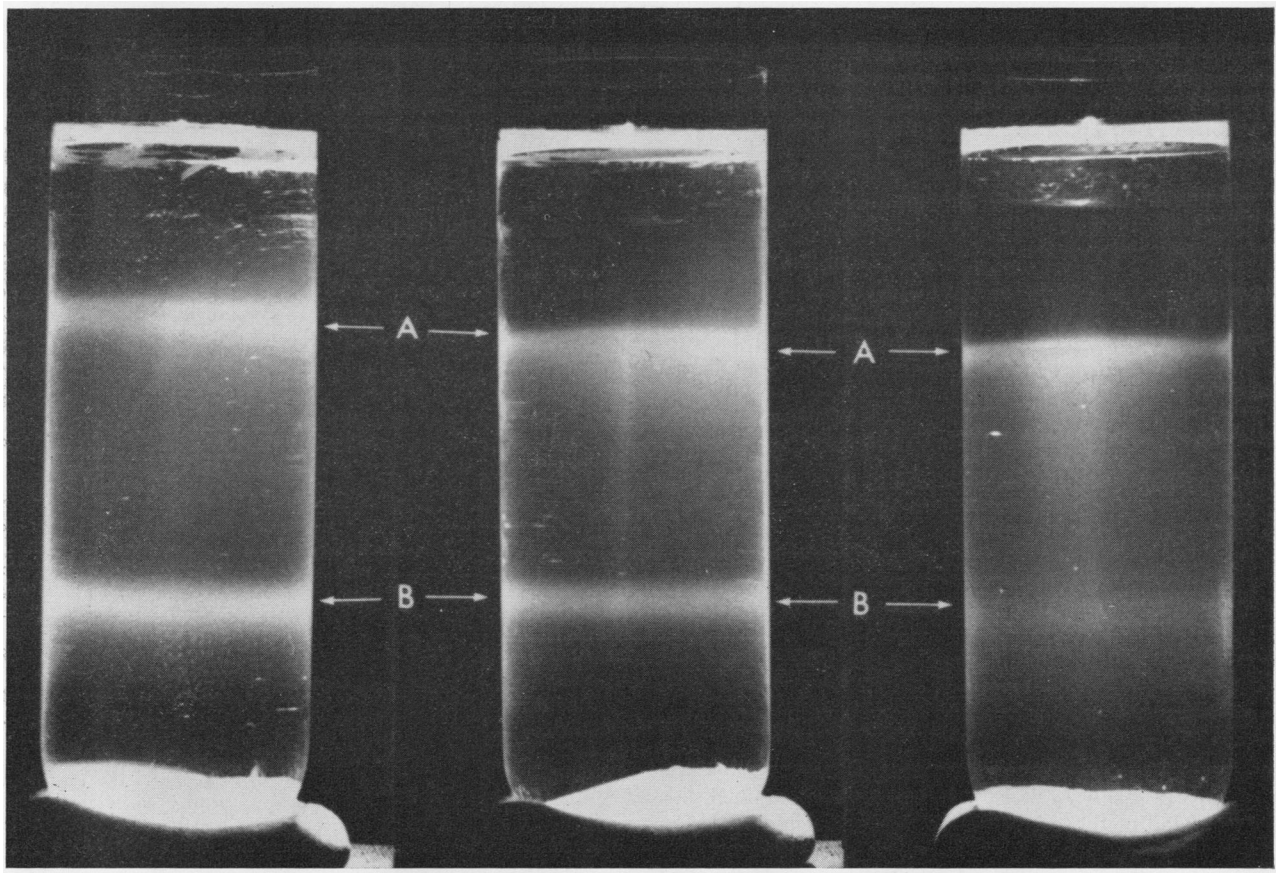

FIG. 6. Sucrose density gradients after 30 min centrifugation at 50,000*g*. Four g of potato tissue slurried in 9 ml total volume were applied to the top of each gradient. Left to right: fresh tissue, potato slices incubated for 2 hr, potato slices incubated for 11 hr. A and B indicate the positions of the two major bands. Potassium phosphate buffer was used.

in tubes 11 to 15 which correspond to the succinoxidase-containing band.

The respiratory rates of fresh and 9-hr-old slices were compared, and in a parallel experiment the succinoxidase activities of isolated mitochondria from fresh and 9-hr-old slices were also compared (Table IV). The results clearly show that, while there is a moderate increase in the succinoxidase activity of isolated mitochondria, there is a striking increase in the respiration of whole slices during the same period.

DISCUSSION

Jackman and Van Steveninck (13, 23) reported that slicing of beet root parenchyma tissue causes damage to mitochondria and endoplasmic reticulum and suggested that during the subsequent aging period mitochondria and endoplasmic reticulum tend to recover their integrity. We have noticed that mitochondria from the fresh tissue migrate as a sharp band on the sucrose gradient, while mitochondria obtained from slices aged 2 or 11 hr (or even "fresh slices") tend to spread (Figs. 3 and 6). Finally, after 26 hr, the succinoxidase band seems to become sharper again (Fig. 3). The spreading of the mitochondrial band due to slicing and aging is consistent with the hypothesis that organelle breakdown and resynthesis are taking place. The Nagoya group has also reported that mitochondrial particles obtained from aged tissue behave differently than do mitochondria from fresh tissue on sucrose density gradient (18, 20, 21).

The very marked decrease in glucose 6-phosphatase activity from 0 to 11 hr of aging might also be interpreted, in the light of Jackman and Van Steveninck (13), as indicating a breakdown of the endoplasmic reticulum, caused or initiated by the mechanical injury.

Table IV. *Changes with Aging of Respiratory Activity in Whole Potato Slices and Isolated Mitochondrial Pellets*

The crude mitochondrial fraction sedimenting between 100*g* and 10,000*g* was extracted from 10 g of potato slices by a modification of Verleur's method (24). The respiratory rate of 30 whole slices (about 1.5 g) and the succinoxidase activity of the isolated mitochondria were measured polarographically as described in "Material and Methods."

	Respiration Rate	
	Whole slices	Mitochondria
	nmoles O_2/min	
Experiment 1		
Fresh	26.3	48.0
9-hr-old	82.5	78.8
Percentage of increase	212	64
Experiment 2		
Fresh	20.0	25.0
9-hr-old	50.0	27.5
Percentage of increase	150	10

The pattern of radioactive choline incorporation into membranes (Fig. 5) changes with the age of the tissue. In freshly cut slices, a major portion of the lipid-soluble radioactivity is

found in the tubes 1 to 5, the "microsomal" zone, which contains most of the glucose 6-phosphatase activity.

In slices aged for 24 hr before the 2-hr incubation, a considerable proportion of the lipid-soluble radioactivity was found in tubes 11 to 16, the "mitochondrial" zone of the gradient by the succinoxidase criterion.

In aged slices, a major portion of the lipid-soluble radioactivity was found in a well defined band intermediate between the microsomal and the mitochondrial zones (band A, in Figs. 1, 2, 5, and 6). Prolonged centrifugation (5.5 hr at 24,500 rpm) drove the band further into the sucrose gradient, peaking at a buoyant density of approximately 1.115 g/cc (Fig. 2). Fresh potato tissue was extracted in the presence of 0.1% glutaraldehyde as a stabilizing agent (17). Bands A and B were collected from the gradient with a hypodermic syringe, fixed in 4% glutaraldehyde, poststained with OsO_4, dehydrated, embedded, and sectioned. Under the electron microscope, band A was revealed to consist of circular profiles of membranous vesicles 1 to 2.5 μ in diameter. The membranes were always single; frequently, two adjacent profiles had one membrane in common. No recognizable mitochondria, proplastids, or endoplasmic reticulum fragments were detected. Band B revealed mitochondria together with some unidentified membranous structures and possibly proplastid fragments.

Morré (16) found that ^{14}C-choline incorporation into lipids is carried out preferentially by dictyosomes in onion stem explants; the same thing appears to be possible in potato slices. Further studies will hopefully reveal whether our band A is derived from disrupted Golgi apparatus. Whatever the cytological nature of band A, it is worth stressing that its lipids are labeled ahead of those in the mitochondrial band. It is too early to speculate whether there is a precursor-product relationship between the lipids of band A membranes and those of mitochondria, although this is, of course, an exciting possibility.

Because of the relative instability of the membrane lipids labeled *in vivo* with ^{14}C-choline, we did not attempt to purify the membrane fractions by isopycnic gradient centrifugation, except as shown in Figure 2. Prolonged manipulations of the membrane fractions cause such great losses of lipid radioactivity that the results become difficult to interpret.

We have previously shown that ^{14}C-choline is used to form lecithin in this system (22). The radioactive lipids in the membrane pellets do not exchange with unlabeled lecithin micelles and can be stabilized simply by boiling. It appears that the radioactive lipids in the isolated membranes are destroyed by the action of enzymes that we have been unable to remove by washing and therefore consider to be also membrane-bound. On the other hand, so long as the slices are intact, the lipoidal radioactivity derived from ^{14}C-choline is stable for many hours (22).

Synthesis of mitochondria during aging has been observed by several workers (2, 15, 25). It was found that after 24 hr of aging there was an increase in the population of mitochondria without any change in their specific enzymatic activity, and this increase was used to explain the respiratory increase in aged slices. Our own results (Table IV) suggest, on the other hand, that, at least during the first 9 hr, factors other than the synthesis of new mitochondrial particles influence the observed respiratory increase.

In conclusion, before one can detect any evidence for the biogenesis of new mitochondria by either labeling studies, direct particle counts, or respiratory measurements, one can demonstrate transformations (biogenesis or turnover) in other membrane systems which result in the incorporation of ^{14}C-choline into membrane-bound lecithin. It will be the object of future investigations to determine the connection, if any, between the turnover of these rapidly labeled membranes and the subsequent biogenesis of mitochondria.

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