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In Vitro Study of Mitochondrial Protein Synthesis during Mitochondrial Biogenesis in Excised Plant Storage Tissue[1]

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BRIAN G. FORDE, ROBIN J. OLIVER, AND CHRISTOPHER J. LEAVER
Department of Botany, The King's Buildings, University of Edinburgh, Mayfield Road, Edinburgh, EH9 3JH, Scotland

ABSTRACT

Mitochondrial biogenesis was induced in Jerusalem artichoke (*Helianthus tuberosus*) tuber by aging tissue discs in distilled water for up to 26 hours. Changes in the purified mitochondrial fraction during aging included an increase in both protein content and specific respiratory activity. Using intact isolated mitochondria, conditions were optimized for incorporation of radioactive amino acid into protein. Incorporation was dependent upon the supply of an oxidizable substrate or an external ATP-generating system and showed characteristic sensitivity to inhibitors of protein synthesis. Aging of the tissue resulted in a 3-fold increase in the rate of *in vitro* incorporation of [^{35}S]methionine into mitochondrial protein. An analysis of the free amino acid pool in the mitochondrial fraction showed that the decrease in methionine level during aging of intact tissue was sufficient to account for the increased rate of protein labeling. The activation of mitochondrial biogenesis which occurs after slicing is not dependent on an increase in the capacity of mitochondria to synthesize protein as assayed *in vitro*.

Analysis, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography, showed that isolated mitochondria synthesized about 15 polypeptides in the molecular weight range 8,000 to 55,000. As aging proceeded, significant changes were observed in the relative rates of labeling of three out of the eight major polypeptides synthesized by mitochondria *in vitro*.

The biogenesis of a functional mitochondrion requires the cooperative expression of both mitochondrial and nuclear genomes. From work mainly on fungal and mammalian cells it has been established that about 90% of mitochondrial proteins, specifically those of the outer membrane, the matrix space, and a large proportion of the proteins of the inner membrane, are encoded in nuclear DNA (12, 19). These proteins are synthesized on cytoplasmic ribosomes and subsequently transferred into the mitochondrial structure by an, as yet, unknown mechanism. The remaining proteins are coded on mitochondrial DNA and synthesized within the mitochondrion on procaryotic-type ribosomes. Recent studies reveal that the majority of these mitochondrial translation products are subunits of enzyme complexes that also contain polypeptides translated on cytoplasmic ribosomes. To date, three of the seven subunits of Cyt oxidase, two to four of the 10 subunits of oligomycin-sensitive ATPase, and one subunit of the Cyt bc_1 complex have been identified as mitochondrial translation products (12, 19). This dual origin of the subunits for some of the important respiratory enzymes suggests the coordination of mitochondrial and cytoplasmic protein synthesis at the level of individual enzymes.

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We are interested in studying the coordination of synthesis and assembly of those proteins synthesized by mitochondria during developmental changes in mitochondrial activity which occur in higher plants. Particular emphasis is placed on the factors controlling mitochondrial biogenesis and turnover, and the way in which biogenesis is linked to the mitochondrion's main metabolic function of respiration and is geared to the development of the plant cell. In the present investigation we have studied mitochondrial protein synthesis during mitochondrial biogenesis in wounded plant storage tissue.

Slicing and aging of plant storage tissue under moist, aerobic conditions induce, in the absence of cell division, a rapid increase in the rate of O_2 uptake and a process of active mitochondrial biogenesis (10, 20). The increase in mitochondrial numbers, as well as in mitochondrial protein and enzyme levels, depends on the activity of both the cytoplasmic (6, 7, 17, 18) and the mitochondrial (2, 16–18) protein-synthesizing systems. In order to distinguish between changes in the rate of protein synthesis that are intrinsic to the organelle itself and those that are under the direct control of cytoplasmic factors, we have isolated and purified the mitochondria from aging tissue prior to testing their ability to incorporate radioactive amino acids into protein.

MATERIALS AND METHODS

Plant Material. Tubers of Jerusalem artichoke (*Helianthus tuberosus*) from a single clone were harvested between November and January and stored in moist sand at 4 C for up to 5 months. Before use the tubers were washed, surface-sterilized in 0.5% sodium hypochlorite at 4 C for 10 min, and rinsed thoroughly with cold sterile distilled H_2O. For aging experiments, 8-mm-diameter cylinders of tissue were removed with a cork borer and sliced into 1.5-mm-thick discs. The discs, in 50-g lots, were washed three times in sterile distilled H_2O and then covered with 50 ml of sterile distilled H_2O in a 250-ml conical flask. Discs that had been stored in ice-cold water for less than 30 min before homogenization were used as "fresh" tissue. Aging tissue was kept aerobic by shaking at 130 cycles/min in a 25 C water bath under constant illumination. To reduce bacterial contamination the water was changed after the 1st hr, and three more times during the subsequent 26-hr incubation.

Preparation of Mitochondria. The method used for isolation of the mitochondria was based on that of Douce *et al.* (8). All utensils and media were sterilized before use. Fifty g of tissue were disrupted in 60 ml of grinding medium using a Willems Polytron homogenizer, fitted with a 2-cm-diameter probe, and operating at full speed for 7 sec. The grinding medium contained: 0.4 M mannitol, 8 mM cysteine, 0.1% (w/v) BSA, 1 mM EGTA, and 10 mM MOPS (pH 8.8). The homogenate was filtered through four layers of muslin and through a double layer of milk filters (Johnson and Johnson, "Marigold") and centrifuged at 1,000*g* for 15 min. The mitochondria were pelleted from the supernatant at

10,000*g* for 15 min and washed in 20 ml of wash medium containing: 0.4 M mannitol, 0.1% (w/v) BSA, 1 mM EGTA, and 5 mM MOPS (pH 7.8). After a further filtration through a milk filter the mitochondria were pelleted again at 10,000*g* for 15 min.

To purify the mitochondrial fraction further the pellet was resuspended in 4 ml of wash medium and loaded onto a discontinuous sucrose gradient. The gradient was formed by layering 4 ml of 0.6 M sucrose in 1 mM EGTA, 0.1% (w/v) BSA, 10 mM Tricine buffer (pH 7.2) onto 2 ml of 1.45 M sucrose in the same buffer. The gradient was centrifuged at 30,000*g* for 30 min in an MSE swing-out rotor (6 × 14 ml) and the mitochondria, which banded at the interface between the two layers of sucrose, were removed using a bent Pasteur pipette. The purified mitochondrial suspension was diluted slowly to an osmolarity of 0.6 M with 0.2 M mannitol, 1 mM EGTA, 10 mM Tricine (pH 7.2). The mitochondria (1–2 mg of protein) were finally pelleted at 10,000*g* for 15 min and resuspended in approximately 200 μl of 0.4 M mannitol, 1 mM EGTA, 10 mM Tricine (pH 7.2).

Duplicate aliquots of the mitochondrial suspension were used for protein determination. After precipitating and washing with 4% (w/v) trichloroacetic acid the protein was solubilized in 0.4 N NaOH and assayed by the method of Lowry *et al.* (13).

For estimation of the density of the mitochondria a continuous density gradient was prepared by layering sucrose solutions in the following sequence: 1.8 M (1.0 ml); 1.45 M (2.5 ml); 1.2 M (2.5 ml); 0.9 M (2.5 ml), and 0.6 M (1.5 ml). The gradient was then allowed to stand at 4 C for 24 hr before use.

Measurement of Respiration Rate. The rate of O_2 uptake by tuber discs was measured manometrically in a Warburg vessel containing 2 ml of sterile distilled H_2O and, in the center well, 0.4 ml of 10% (w/v) KOH. Respiration was followed at 25 C for approximately 50 min with shaking at the rate of 100 cycles/min.

The respiratory activity of the isolated mitochondria was assayed at 20 to 22 C with a Clarke O_2 electrode. The assay medium, which was aerated before use, contained: 0.4 M mannitol, 10 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.1% (w/v) BSA, and 10 mM K-phosphate (pH 7.2).

Amino Acid Incorporation. The purified mitochondria (250–500 μg of protein) were incubated in test tubes (5 × 1 cm) in a final volume of 250 μl. The basic incubation medium contained 0.25 M mannitol, 90 mM KCl, 10 mM Tricine buffer (pH 7.2), 5 mM K-phosphate (pH 7.2), 1 mM EGTA, and 25 μM amino acid mixture (minus methionine). In addition one of the following mixtures was included: (*a*) 10 mM succinate, 2 mM ADP, 2 mM GTP, and 30 mM $MgCl_2$ (when ATP was to be generated by oxidative phosphorylation), or (*b*) 8 mM creatine phosphate, 100 μg/ml creatine phosphokinase, 6 mM ATP, 0.5 mM GTP, and 7.5 mM $MgCl_2$ (when ATP was to be generated externally). L-[^{35}S]Methionine (530–630 Ci/mmol) was supplied at a concentration of 8 to 80 μCi/ml as required. Throughout the incubation the tubes were shaken at approximately 300 cycle/min and maintained at 25 C.

The time course of the reaction was routinely followed by removing 20-μl samples onto 1.5-cm square filter paper discs. The discs were dried in a stream of air for 30 sec before they were immersed in ice-cold 10% (w/v) trichloroacetic acid for at least 15 min. Radioactivity not incorporated into protein was removed by a series of washes (14): in 5% trichloroacetic acid at 90C for 15 min; in 5% trichloroacetic acid at room temperature for 5 min (four times); in ethanol-ether (1:1) at 37 C for 15 min; and finally in ether at 37 C for 10 min. The discs were dried again and the radioactivity remaining was counted in a scintillation counter with an efficiency of 70% in scintillant containing 4 g of 2-(4-6-butylphenyl)-5-(4″-biphenyl)-1,3,4-oxadiazole/l toluene.

Bacterial contamination was monitored by plating samples of incubation medium or mitochondrial suspension onto 2.8% (w/v) nutrient agar. The number of colonies which developed after 48 hr at 25 C were always less than 2,000/incubation. We have found that at least 10^5 bacteria are required per incubation to contribute significantly to the incorporation of [^{35}S]methionine.

Polyacrylamide Gel Electrophoresis. Following amino acid incorporation, mitochondrial protein in the incubation medium was dissociated by adding 0.33 volumes of a buffer containing 8% (w/v) sodium dodecyl sulfate, 20% (v/v) β-mercaptoethanol, 40% (w/v) sucrose, and 0.25 M Tris-HCl buffer (pH 6.8). The mixture was heated to 37 C for 30 min. The polypeptides were fractionated on a 15% (w/v) polyacrylamide slab gel with a 5% stacking gel using the discontinuous buffer system described by Laemmli (11). A number of marker proteins were used in the estimation of the mol wt of the dissociated polypeptides according to the method of Weber and Osborn (21): BSA (mol wt, 68,000), pyruvate kinase (57,000), large subunit of fraction 1 protein (55,500), creatine phosphokinase (40,000), alcohol dehydrogenase (24,000), trypsin inhibitor (21,000), small subunit of fraction 1 protein (12,000), and Cyt *c* (11,700). The gels were electrophoresed overnight at a constant current of 10 ma/gel, stained with Coomassie blue, and dried onto Whatman 3MM chromatography paper. The dry gels were exposed to Kodak Blue Brand x-ray film for up to 2 weeks. The autoradiographs were scanned using a Kipp and Zonen microdensitometer with a 0.1-mm slit width setting.

Measurement of [^{35}S]Methionine Uptake. The mitochondria were incubated under the usual conditions for amino acid incorporation and then pelleted by centrifugation at 8,000*g* for 5 min. The pellet was solubilized in 0.5 ml of 0.5 N NaOH at 37 C for 15 min. After addition of 0.2 ml of 50% (w/v) trichloroacetic acid the samples were allowed to stand at 0 C overnight. The trichloroacetic acid precipitate was pelleted at 8,000*g* for 3 min and washed twice with 0.5 ml of 5% trichloroacetic acid. The three supernatants (the "trichloroacetic acid-soluble fraction") were pooled. The final pellet (the "trichloroacetic acid-insoluble fraction") was hydrolyzed in 0.5 ml of 0.5 N NaOH at 45 C for 60 min and an aliquot was taken for protein determination. Duplicate 20-μl samples of both fractions were dried onto filter paper discs for scintillation counting.

Analysis of Amino Acid Pool. Approximately 3 mg of gradient-purified mitochondrial protein was resuspended in 0.4 ml of ice-cold 2.5% (w/v) trichloroacetic acid containing 2% (v/v) thiodiglycol (to prevent oxidation of methionine). After 60 min the trichloroacetic acid precipitate was pelleted at 8,000*g* for 3 min and the supernatant was carefully removed with a Pasteur pipette. The pellet was extracted twice more with 0.2 ml of 2.5% trichloroacetic acid-2% thiodiglycol. The pooled supernatants were taken to dryness in a rotary evaporator, dissolved in sterile H_2O, and the amino acid content analyzed on a Beckman 120 C amino acid analyzer using the methodology of Benson and Patterson (3).

RESULTS

Aging and Respiratory Activity. Figure 1 illustrates the effect of aging on the respiration of Jerusalem artichoke tuber discs. A rapid increase in the rate of O_2 uptake by the discs of tissue was induced after slicing, and for the first 14 hr this occurred in the absence of any change in the respiratory activity of the isolated mitochondrial fraction. In other plant storage tissues a similar increase in O_2 uptake immediately after slicing, which can occur independently of RNA and protein synthesis, has been attributed to oxidation of fatty acids (10, 20) or to the activation of glycolysis by adenine nucleotides (1).

Later in the period of aging, between 14 and 26 hr after slicing, there was an almost 2-fold increase in the respiratory activity of the mitochondrial fraction. The data in Table I suggest that there are two components to this change, an increase both in the specific respiratory activity (expressed per mg mitochondrial protein), and in the yield of mitochondrial protein. The increases observed in specific respiratory activity and in protein yield during the 26 hr of aging were consistently greater than 20 and 36%, respectively.

The respiratory control and ADP/O ratios presented in Table I demonstrate that aging has no effect on the integrity of the isolated mitochondria or on the efficiency of oxidative phospho-

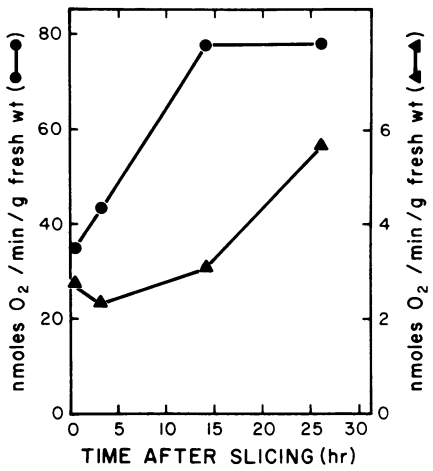

FIG. 1. Increase in respiration rate during aging of Jerusalem artichoke tuber discs. Rates of O_2 uptake by tissue discs (●——●) and by isolated mitochondrial fraction (▲——▲) are each expressed/g fresh weight of tuber tissue.

Table I. Changes in the respiratory properties of the mitochondrial fraction during aging of artichoke tuber discs.

The respiratory activity of the purified mitochondria was assayed in the O_2 electrode as described in Methods. The values given are averages and the number of individual experiments in which each was determined is shown in parentheses.

	Time after slicing (hr)			
	0	3	14	26
Protein content (μg/g fresh weight of tissue)	31.0(5)	30.0(2)	42.2(2)	50.0(5)
Specific respiratory activity (nmoles O_2/min/mg protein)	91.0(5)	75.4(2)	73.1(2)	114.3(6)
Respiratory control ratio	3.1(8)	3.6(3)	3.0(3)	3.2(7)
ADP/O ratio	1.68(8)	1.68(3)	1.69(3)	1.66(7)

rylation. The values given for respiratory control refer consistently to the third cycle of ADP addition since it was found with mitochondria from both fresh and aged tissue that the initial state 3 rate was not linear and that successive additions of ADP gave faster state 3 rates (Fig. 2). Unlike a similar phenomenon which has been observed with potato mitochondria (22) the lag could not be eliminated by preincubation with ATP.

In contrast to the many reports that an alternative cyanide-insensitive electron transport pathway develops during aging of other storage tissues (10, 20), respiration by mitochondria from both fresh and 26-hr aged discs of artichoke tuber was inhibited more than 85% by 0.1 mM KCN (Fig. 2).

Mitochondrial Density during Aging. Aging of the tuber tissue resulted in a decrease in the buoyant density of the mitochondria in a sucrose gradient (Fig. 3). As there was only one well defined band present at all stages of aging it is likely that the change occurred in existing mitochondria as well as in those newly formed after slicing. It is not clear whether the decrease in density should be attributed to an increase in the ratio of lipid to protein or simply to an osmotic effect.

Conditions for Amino Acid Incorporation by Isolated Mitochondria. Before comparing the ability of mitochondria from fresh and aged tissue to synthesize protein *in vitro*, we investigated the optimum conditions necessary for amino acid incorporation into protein.

When the mitochondria were incubated in the presence of succinate as the respiratory substrate, [^{35}S]methionine incorporation was found to be strongly dependent on the addition of ADP (Fig. 4A). At all concentrations of ADP, there was an initial lag period in the incorporation of label. This lag may be associated with the delay, noted in Figure 2, before the maximum state 3 respiration rate is attained. Incorporation of [^{35}S]methionine began without delay when ATP was generated externally by an enzyme system in the incubation medium (see Fig. 5B) or when the mitochondria were preincubated with succinate and ADP (not shown).

Incorporation of [^{35}S]methionine also depended on the presence of Mg^{2+} (Fig. 4B). The concentrations of ADP and Mg^{2+} chosen for use in later experiments, 2 mM and 30 mM, respectively, were those which gave a high level of incorporation with the minimum initial lag period.

ATP generated from substrates other than succinate can support [^{35}S]methionine incorporation into protein (Table II). Substitution of malate for succinate had no significant effect on the rate of labeling. When acetate, which cannot serve as a respiratory substrate, was used instead of succinate, only a very low rate of incorporation was obtained. ATP generated outside the mitochondria, using creatine phosphate and creatine phosphokinase or P-enolpyruvate and pyruvate kinase, was able to sustain *in vitro* protein synthesis at a lower rate than when energy was generated by oxidative phosphorylation.

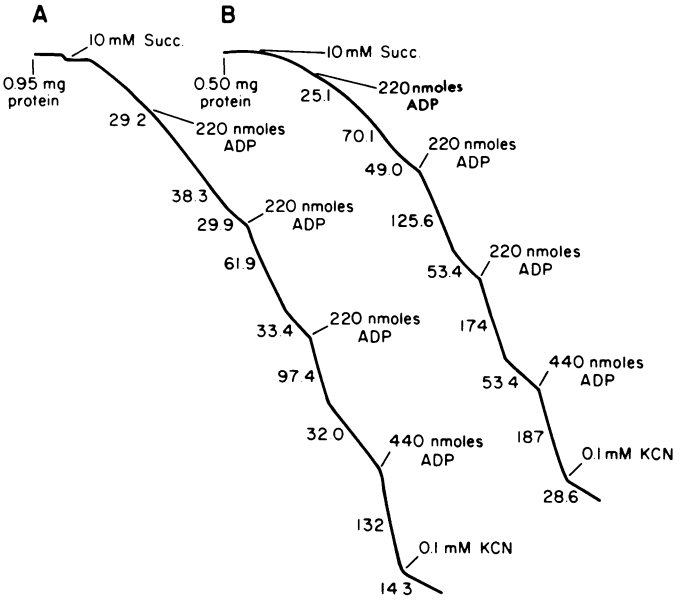

FIG. 2. O_2 electrode traces obtained with mitochondria from fresh and aged tissue. Mitochondrial fraction from (A) fresh and (B) 26-hr aged tissue was purified on a discontinuous sucrose gradient and assayed for respiratory activity. Figures to the left of the traces indicate the rate of O_2 uptake in nmol/min·mg protein.

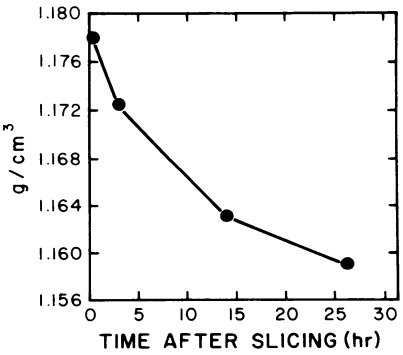

FIG. 3. Decrease in mitochondrial density during aging. Mitochondria, isolated by differential centrifugation, were centrifuged for 90 min at 40,000g_{av} in a continuous sucrose gradient. Density of sucrose at which the organelles banded was determined by refractometry.

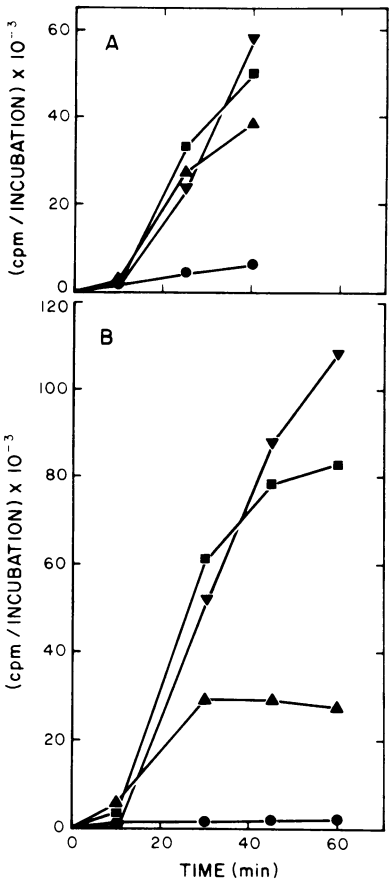

FIG. 4. Effect of ADP and Mg^{2+} concentrations on time course of [^{35}S]methionine incorporation into protein by isolated mitochondria. Gradient-purified mitochondria from fresh tissue were incubated at 25 C in 250 μl of the basic incubation medium. In addition the mixture contained, in (A): 10 mM succinate, 2 mM GTP, 15 mM $MgCl_2$, 2.5 μCi of [^{35}S]-methionine and either no ADP (●——●), 1 mM ADP (▲——▲), 2 mM ADP (■——■), or 5 mM ADP (▼——▼), and in (B): 10 mM succinate, 2 mM GTP, 2 mM ADP, 2.5 μCi of [^{35}S]methionine, and either no $MgCl_2$ (●——●), 5 mM $MgCl_2$ (▲——▲), 15 mM $MgCl_2$ (■——■), or 30 mM $MgCl_2$ (▼——▼). At intervals during the incubation 20-μl samples were removed and the amount of radioactivity incorporated into protein was assayed as detailed under "Materials and Methods."

Table II. Effect of different energy sources on the rate of [^{35}S]-methionine incorporation into protein.

Mitochondria were incubated in the basic incubation medium containing 2mM GTP, 30mM $MgCl_2$ and 2.5 μCi[^{35}S]-methionine, with the addition, where indicated, of the following: ADP, 2mM; ATP, 6mM; succinate, malate and acetate, 20mM; creatine phosphate, 8mM; phosphoenol pyruvate, 5mM; creatine phosphokinase, 100 μg/ml and pyruvate kinase, 20 μg/ml. [^{35}S]-methionine incorporation after 40 min incubation is expressed as a percentage of that obtained with succinate and ADP (4.5×10^4 cpm/incubation).

Energy source	Radioactivity incorporated (% of control)
ADP	
+succinate	100
+malate	97.2
+acetate	11.8
Phosphoenol pyruvate + pyruvate kinase + ATP	22.6
" " " " - ATP	3.2
Creatine phosphate + creatine phosphokinase + ATP	27.5
" " " " - ATP	6.0
ATP alone	19.2

A number of properties of this *in vitro* protein-synthesizing system, notably the dependence on the presence of Mg^{2+} and ADP and the failure of acetate to support a significant rate of incorporation, indicate that the observed low level of bacterial contamination (see under "Materials and Methods") plays a negligible part in the reaction.

Effect of Aging on the Rate of Amino Acid Incorporation into Mitochondrial Protein. During the first 26 hr after slicing, the rate of *in vitro* [^{35}S]methionine incorporation per mg mitochondrial protein increased 3-fold (Fig. 5). The increase was similar whether ATP was provided by oxidative phosphorylation (Fig. 5A) or by an external energy generating system (Fig. 5B) and therefore cannot be attributed to an increase in respiratory activity.

Membrane Permeability and Rate of [^{35}S]Methionine Incorporation into Protein. The permeability of mitochondria to methionine is low (9) and since the biosynthesis of this amino acid can take place within the matrix of higher plant mitochondria (5) it is unlikely that a mechanism would exist for its active uptake into the organelle. Any changes in the properties of the mitochondrial membranes which enhance [^{35}S]methionine uptake might therefore have a very marked effect on the rate of labeling of protein *in vitro*. Measurement of the level of radioactive amino acid in the mitochondrial pool revealed no significant difference between mitochondria isolated from fresh and from 26-hr aged tissue (Table III). In each case about 0.4 pmol of methionine/mg protein (or about 3% of that supplied in the medium) was present in the trichloroacetic acid-soluble pool at the end of the incubation, regardless of whether ATP was generated endogenously or externally.

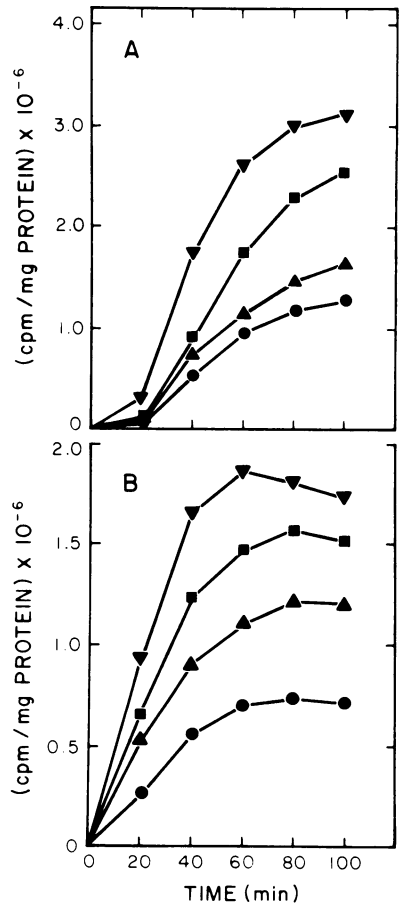

FIG. 5. Effect of aging on rate of [^{35}S]methionine incorporation by isolated mitochondria. Mitochondria from fresh (●——●), 3-hr aged (▲——▲), 14-hr aged (■——■), and 26-hr aged (▼——▼) tissue discs were incubated in basic incubation medium with the addition of (A) succinate, ADP, GTP, and $MgCl_2$ or (B) creatine phosphate, creatine phosphokinase, ATP, GTP, and $MgCl_2$ at the concentrations indicated under "Materials and Methods." Concentration of [^{35}S]methionine (635 Ci/nmol) in (A) was 80 μCi/ml and in (B) was 120 μCi/ml.

To investigate the further possibility that the aging process affects the uptake of a component of the incubation medium other than [^{35}S]methionine, we employed a nonionic detergent, Triton X-100, in an attempt to remove selectively barriers to permeability without destroying the protein synthetic capacity of the mitochondria. A detergent concentration of 0.03% (w/v) was found sufficient to eliminate completely the respiratory activity of the mitochondria from both fresh and aged tissue (Fig. 6). Even at a Triton X-100 concentration of 0.1%, the rate of [^{35}S]methionine incorporation into protein by mitochondria from 26-hr aged tissue remained 150% higher than by those from fresh tissue (Fig. 6). It is unlikely that a change in mitochondrial membrane permeability after slicing is responsible for the increased rate of incorporation after aging.

Unexpectedly, concentrations of Triton X-100 up to 0.04% stimulated the rate of protein labeling by as much as 500% (Fig. 6). This effect could be explained if these detergent levels were allowing free exchange between the endogenous methionine pool and the [^{35}S]methionine in the medium. Inasmuch as the mitochondria normally take up only a small fraction of the added radioactive methionine (Table III), this mixing would result in a greatly increased specific radioactivity at the site of mitochondrial protein synthesis. The strong inhibition which was observed at the higher detergent concentrations may be due to dilution of components of the protein-synthesizing system into the incubation medium.

Table III. Uptake of [^{35}S]-methionine by mitochondria from fresh and aged tissue.

Mitochondria were incubated for 90 min in the basic incubation medium containing 2mM GTP, 30 mM $MgCl_2$, 2μCi [^{35}S]-methionine (530 Ci/nmole) and the appropriate energy source. They were then pelleted by centrifugation, and the trichloroacetic acid-soluble and trichloroacetic acid-insoluble radioactivity was estimated as described in Methods. [a].The figures in parentheses are the levels of [^{35}S]-methionine accumulated in the trichloroacetic acid-soluble pool expressed as a percentage of the total activity supplied (2.8×10^6 cpm/incubation).

Time after slicing (hr)		Radioactivity incorporated (cpm/mg protein) x 10^{-3} Trichloroacetic acid insoluble	soluble
0	ADP + succinate	132	430(3.3)[a]
	ATP + creatine phosphate, creatine phosphokinase	76.6	330(2.5)
26	ADP + succinate	492	360(2.7)
	ATP + creatine phosphate, creatine phosphokinase	290	470(3.5)

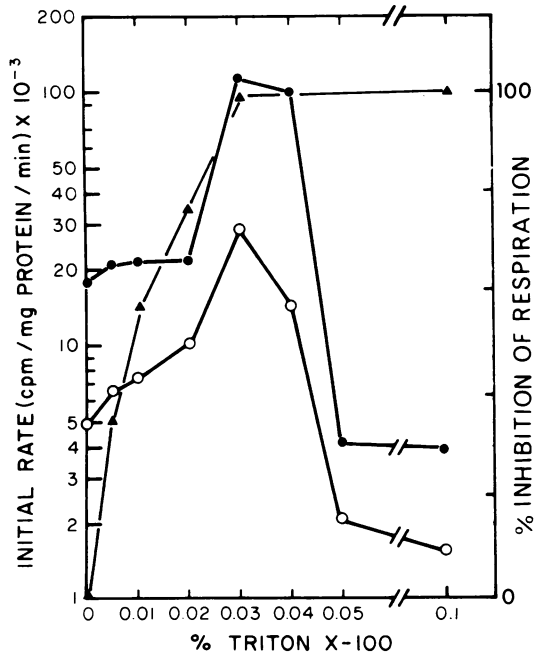

FIG. 6. Effect of a range of concentrations of Triton X-100 on rate of incorporation of [^{35}S]methionine into protein by mitochondria from fresh and aged tissue. Mitochondria from freshly sliced (○——○) and from 26-hr aged (●——●) tissue were incubated in a complete incorporation medium containing 20 μCi of [^{35}S]methionine and various concentrations of Triton X-100. Concentration of mitochondrial protein in all incubations was 1.6 mg/ml and energy was supplied by a creatine phosphokinase energy generating system. State 3 respiration was measured in the O_2 electrode with increasing concentrations of Triton X-100 (▲——▲).

Amino Acid Pool Size and Rate of [^{35}S]Methionine Incorporation into Protein. A decrease in the intramitochondrial concentration of methionine would result in an increase in the rate of incorporation of [^{35}S]methionine in the *in vitro* assay. If this was occurring during aging it should be possible to reduce, or even eliminate, the difference between mitochondria from fresh and aged tissue simply by assaying the incorporation of [^{35}S]methionine in the presence of high concentrations of unlabeled methionine. The effect on the incorporation rate of progressively decreasing the specific radioactivity of the amino acid, while keeping the level of radioactivity constant, is shown for mitochondria isolated from fresh and aged tissue in Table IV. The experiment was performed in the presence of 0.03% Triton X-100 in an attempt to reduce as far as possible membrane barriers to mixing of internal and external pools of methionine. It can be seen that as the concentration of unlabeled methionine in the medium was increased, the rate of labeling was reduced to a much greater extent in mitochondria from 26-hr aged tissue than in those from fresh tissue. Thus at the lowest specific radioactivity, when the concentration of methionine added was 100 μM (100 nmol/mg protein), the rate of incorporation by mitochondria from the aged tissue was only 47% greater than that by mitochondria from fresh tissue, while at the highest specific radioactivity the difference was 970%. Similarly, when [^{14}C]leucine or a [^{14}C]protein hydrolysate were supplied instead of the [^{35}S]methionine, at low specific activities, there was no significant difference in the rate of labeling shown by the two types of mitochondria (Table IV).

The results in Table IV could be most simply explained by a depletion of the mitochondrial amino acid pool during aging. Amino acid analysis of the mitochondrial trichloroacetic acid-soluble fraction confirms that changes in the amino acid levels do occur which could account for the observed increase in the rate of *in vitro* [^{35}S]methionine incorporation into protein (Table V). Not only the methionine pool but also that of each of the other amino acids measured was several times greater in mitochondria isolated from fresh tissue than in mitochondria obtained from tissue that had been aged for 26 hr. The same response to aging has been

Table IV. Effect of isotope dilution on the rate of incorporation of labelled amino acid by mitochondria from fresh and aged tissue.

Mitochondria (250 μg protein) were incubated in 250 μl of the basic incubation medium containing 0.03% Triton X-100, the appropriate radioactive amino acid and the energy generating mixture described in Methods. A range of methionine concentrations was obtained by adding different amounts of unlabelled methionine. When the mitochondria were incubated with [^{35}S]-methionine (20 μCi) or [^{14}C]-leucine (25 μCi) they were supplied with a mixture of 19 unlabelled amino acids (25 μM) lacking the radioactive amino acid; when the incubation was with [^{14}C]-protein hydrolysate (50 μCi) no additional amino acids were included. [a].Because the time course of the incorporations was slightly altered by changing the amino acid concentration, the initial rate of labelling (during the first 10 min of incubation) was chosen as the means of comparison. The general pattern, however, would not have been significantly affected by using a longer time period. [b].The concentration of the [^{14}C]-protein hydrolysate is expressed as natoms of carbon/ml.

	Final concentration of carrier amino acid (μM)	Initial rate of incorporation [a]. (cpm/mg protein/min x 10^{-3}) fresh	26 hr aged	% increase
[^{35}S]-methionine	0.12	43.4	464	969
	0.62	22.8	80.8	254
	2.6	10.3	31.5	206
	12.6	3.77	8.64	129
	25.1	2.63	5.50	109
	100	1.80	2.64	47
[^{14}C]-leucine	290	5.50	5.57	1.3
[^{14}C]-protein hydrolysate	96.8 [b]	13.3	13.8	3.8

Table V. Amino acid composition of the trichloroacetic acid-insoluble and trichloroacetic acid-soluble fractions of mitochondria from fresh and aged tissue.

The mitochondria were isolated from fresh and 26hr aged tissue discs and purified in the usual way on a discontinuous sucrose gradient. The trichloroacetic acid-soluble and trichloroacetic acid-insoluble fractions were prepared for amino acid analysis as described in Methods.

	Concentration (nmoles/mg protein)			
	Trichloroacetic acid			
	insoluble		soluble	
	Fresh	26hr aged	Fresh	26hr aged
Aspartic acid	464	448	3.4	0.88
Threonine	230	199	1.9	0.25
Serine	252	262	10.0	5.7
Glutamic acid	434	450	2.50	1.1
Proline	28	28	ND	ND
Glycine	420	426	4.7	0.30
Alanine	416	422	2.2	0.60
Valine	292	308	1.9	0.40
Methionine	60	68	0.19	ND
Isoleucine	232	240	1.30	ND
Leucine	426	432	1.60	ND
Tyrosine	122	120	ND	ND
Phenylalanine	180	188	0.63	ND

noted for the amino acid pool of the whole cell in a variety of storage tissues (10).

Products of *in Vitro* Protein Synthesis by Mitochondria from Fresh and Aged Tissue. The polypeptides radioactively labeled by isolated mitochondria were analyzed by polyacrylamide gel electrophoresis in SDS, followed by autoradiography of the dried slab gels. Densitometer traces of the autoradiographs (Fig. 7) show that eight major polypeptides, in the mol wt range 8,000 to 55,000, were labeled *in vitro*. In some experiments not reported here, when the level of bacterial contamination was greater than 10^5 organisms/incubation, the contribution of bacterial protein synthesis was characterized by both a greatly increased number of translation products and the appearance of many labeled polypeptides of mol wt greater than 60,000.

A polypeptide pattern similar to those in Figure 7 was obtained with artichoke mitochondria in an earlier report (12), whether the products were labeled *in vivo* in the presence of cycloheximide or *in vitro* as described above.

Although the same major polypeptides are synthesized by mitochondria isolated from tissue throughout the aging process, there are changes in the relative proportions of three of the eight polypeptide bands (Fig. 7). As aging proceeded there was a marked increase in the relative rate of synthesis of two polypeptides with mol wt 17,000 and 34,500, whereas that of a third polypeptide, mol wt 19,000, was reduced.

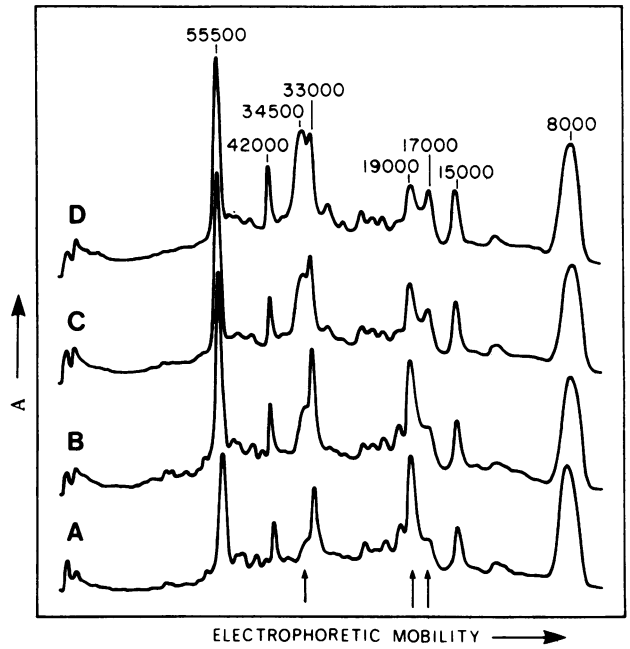

FIG. 7. Effect of aging on products of *in vitro* mitochondrial protein synthesis. At the end of the incubations described in Figure 5, mitochondria were solubilized in 2% SDS and the dissociated proteins were separated by polyacrylamide gel electrophoresis as described under "Materials and Methods." Approximately 1×10^5 cpm incorporated into protein was electrophoresed in each case. An autoradiogram obtained from the dry gel was scanned optically using a microdensitometer with 0.1-mm slit width and the same *A* settings for each sample. Scans shown are for incubations in the presence of an external energy generating system but almost identical traces were obtained in each case when energy was generated by oxidative phosphorylation. Mitochondria used in the incubations were from tissue that had been aged for: (A) 0 hr; (B) 3 hr; (C) 14 hr; and (D) 26 hr.

DISCUSSION

The rapid increase in respiration rate which is induced by slicing and aging of Jerusalem artichoke tuber tissue (Fig. 1) is followed by active biogenesis of the mitochondria, as demonstrated by a significant increase in the protein content of the isolated mitochondrial fraction and by an enhancement of its specific respiratory activity (Table I).

When mitochondria isolated from tissue which had been aged for 26 hr were compared with those from fresh tissue for their ability to synthesize protein *in vitro*, it was found that the aging treatment produced a 3-fold increase in the rate of [^{35}S]methionine incorporation into protein (Fig. 5). An increase in the ability to incorporate radioactive amino acid does not necessarily reflect a real increase in the protein synthetic capacity of the mitochondria. At least two other changes, separately or in combination, could account for this result: (*a*) an increase in mitochondrial membrane permeability, allowing *e.g.* an increased uptake of [^{35}S]methionine from the incubation medium; or (*b*) a decrease in the pool size of methionine in the isolated mitochondria. Either of these effects would give rise to an increase in the specific radioactivity of the amino acid in the matrix of the organelle.

Inasmuch as the free radioactive amino acid pool does not change significantly in mitochondria from fresh and aged tissue (Table III), it is unlikely that the first alternative can explain the present results. Even after treatment with low levels of Triton X-100, mitochondria from aged discs still incorporated [^{35}S]methionine more than twice as fast as did those from fresh discs (Fig. 6).

To investigate the second alternative we analyzed the amino acid pools in the isolated mitochondria. This revealed a sharp fall in the methionine level (from 0.2 nmol to less than 0.07 nmol/mg protein) as a result of aging (Table V). [^{35}S]Methionine was normally supplied with only a very low concentration of carrier methionine (0.03–0.12 nmol/mg mitochondrial protein), and because the percentage of this taken up by the mitochondria was both small and independent of the age of the tissue from which they were isolated (Table III), a fall in the endogenous methionine pool of the kind observed would produce a corresponding increase in the specific radioactivity of the amino acid within the mitochondrion. By increasing the methionine concentration in the incubation medium to 100 nmol/mg protein, and so swamping the endogenous pool, we could almost eliminate the difference between the rate of labeling of mitochondria from fresh and aged tissue (Table IV). Thus, a reduction in the size of the endogenous methionine pool could account for the increase in the rate of [^{35}S]methionine incorporation during aging (Fig. 5). A previous report that the protein synthetic capacity of isolated artichoke mitochondria is markedly stimulated by aging of the tissue (4) was based on incorporation experiments which also used low concentrations of radioactive amino acid (0.5 nmol of [^{14}C]leucine/mg protein). We have used [^{14}C]leucine at 290 nmol/mg protein and found that mitochondria from fresh and aged tissue incorporated

labeled amino acid into protein at almost identical rates (Table IV), despite a very marked depletion of the intramitochondrial leucine pool during aging (Table V).

We concluded that aging of artichoke tuber discs has no apparent effect on the ability of the isolated mitochondria to synthesize protein. This does not necessarily imply that mitochondrial protein synthesis is not activated *in vivo* after wounding. It has been shown that chloramphenicol treatment inhibits mitochondrial biogenesis in aging sweet potato root tissue (2, 16–18), suggesting a dependence on protein synthesis in the organelle. It is possible that mitochondrial translation *in vivo* is regulated during aging by cytoplasmic factors which are not carried over into the purified mitochondrial fraction. Work on yeast mitochondria has indicated that synthesis of mitochondrial translation products is controlled in a positive manner by the availability of certain cytoplasmically synthesized proteins which are components of enzyme complexes in the inner membrane (15, 19). Therefore, the renewed synthesis in the cytoplasm of proteins destined for the mitochondrion which occurs after wounding of storage tissue (6, 7, 17, 18) may in turn activate mitochondrial protein synthesis.

Mitochondria isolated from artichoke tuber synthesize about 15 polypeptides which are detectable on SDS-polyacrylamide slab gels (Fig. 7). As yet none of the proteins coded for by plant mitochondrial DNA have been identified, but by analogy with Ascomycetes and mammalian cells it is likely that some are components of the Cyt oxidase, Cyt bc_1, and oligomycin-sensitive ATPase enzyme complexes (12, 19). During aging, changes were observed in the relative rates of synthesis of three out of the eight major mitochondrial translation products (Fig. 7). The detection of these changes in mitochondria which had been isolated and purified suggests that they are a response to aging which is intrinsic to the organelle and not one which is under the direct control of cytoplasmic factors.

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