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Control of Changes in Mitochondrial Activities during Aging of Potato Slices[1]

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ABSTRACT

Aging of slices of potato tuber (*Solanum tuberosum* L.) in an aerated liquid medium induces a number of changes in mitochondrial activities. A nonphosphorylative, cyanide-insensitive electron transport pathway (alternate pathway) is brought into operation. The rate of oxidation of exogenous NADH increases markedly and the efficiency of phosphorylation with this substrate remains the same as it is in mitochondria isolated from fresh tissue slices. On the contrary, the rates of oxidation of succinate and malate do not increase while lower phosphorylative efficiencies indicate that a fraction of their electrons reaches oxygen through the alternate pathway. Chloramphenicol, a specific inhibitor of the mitochondrial protein-synthesizing system, has no effect whatsoever on these events. However, cycloheximide, which acts on the corresponding cytoplasmic system, prevents both the development of the alternate pathway and the rise in the rate of oxidation of exogenous NADH. These effects are interpreted as showing a specific control of the cytoplasmic protein-synthesizing system on the changes in mitochondrial oxidations during aging.

It is now well established that the aging of tissue slices from various tubers, in moist atmospheres or in liquid-aerated media, induces a number of metabolic changes within the tissue (15, 28). In potato slices, aging brings about a large increase in tissue respiration which, moreover, becomes markedly resistant to cyanide or carbon monoxide inhibition (14, 26). The resistance to cyanide which appears during aging has been explained, at the mitochondrial level, by the development of an alternate electron transport pathway which is nonphosphorylative and insensitive to both cyanide and antimycin. This new pathway can be specifically inhibited by derivatives of hydroxamic acid, such as salicylhydroxamic acid (2, 21). In addition, the oxidation patterns of various substrates are also diversely affected by aging (8, 14).

In slices of potato tubers, the aging process is also known to be linked to an active synthesis of new nucleic acids, proteins, and membrane systems (7, 15). The question then arises of whether new enzymes or new electron transport components are synthesized during aging that could account for the respiratory rise in the tissue and for the changes in mitochondrial activities. Furthermore, the use of specific inhibitors of protein synthesis such as chloramphenicol or cycloheximide could yield useful information on the relations between protein synthesis and the development of new mitochondrial properties (20). This approach was used in this work and has led to the conclusion that the cytoplasmic protein-synthesizing system controls the effects of aging at the mitochondrial level.

MATERIALS AND METHODS

Potato tubers (*Solanum tuberosum* L., variety Bintje) were obtained from the Coopérative Agricole of Pleyber-Christ, Finistère, and stored at 4 C until used. Cylinders of tissue were cut out of the central part of the tubers and sliced into discs about 1-mm thick. The discs were then rinsed briefly with cold distilled H_2O and either used immediately as fresh slices or let to age for 24 hr. Aging took place at 25 C in 1-liter Erlenmeyer flasks containing 50 g of tissue slices in 40 ml of 0.1 mM $CaSO_4$. The flasks were aerated by continuous rotatory shaking (180 rpm). To prevent bacterial contamination without interfering with protein synthesis, chloramphenicol (50 μg/ml) was added to the medium. The medium was renewed four times during the first 12 hr of aging. In some experiments, chloramphenicol (350 μg/ml) or cycloheximide (1 μg/ml) was used as inhibitor of protein synthesis.

The preparation of mitochondria from fresh or aged tissue slices was carried out according to the procedures and using the media described by Bonner (4) with only minor modifications. In addition, the mitochondria were purified by centrifugation on sucrose density gradients (four layers of 1.8, 1.5, 1.2, and 0.9 M sucrose containing 0.1% BSA and 10 mM phosphate buffer, pH 7.2). After centrifugation at 35,000g for 50 min, the mitochondria on the top of the 1.5 M layer were collected, diluted, resedimented (10,000g for 15 min), and finally suspended in a small volume of the washing medium.

O_2 uptake was measured at 25 C in a Plexiglas cell fitted with a Clark O_2 electrode in a medium of 300 mM mannitol, 5 mM $MgCl_2$, 10 mM KCl, 10 mM K buffer (pH 7.2), and 1 mg/ml of BSA. O_2 concentration in air-saturated medium was taken as 240 μM (13). Depending on the substrate used (succinate, 10 mM; malate, 30 mM; NADH, 1 mM) 0.5 to 1.5 mg of mitochondrial proteins were used in a final volume of 4 ml. The mitochondria were systematically incubated for 2 min in the presence of 200 μM ATP prior to the addition of substrates (29). ADP/O ratios and respiratory control values were determined according to Estabrook (13). Mitochondrial proteins were measured by acid digestion and nesslerization.

KCN in a water solution neutralized to pH 7.2 and SHAM[3] (Aldrich) dissolved in ethanol were used as inhibitors of electron transport. Chloramphenicol and cycloheximide were obtained from Sigma Chemical Co.

RESULTS

Mitochondrial Changes Induced by Aging. Mitochondria isolated either from fresh or from aged tissue slices oxidize succi-

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[3] Abbreviations: SHAM: salicylhydroxamic acid; RC: respiratory control (state 3 rate of oxidation/state 4 rate of oxidation).

nate at rather similar rates (Fig. 1, A and B; Table I), as shown by state 3 rates of oxidation (5). However, state 4 rates appear to be much higher in mitochondria from aged tissue. As a result, a decrease in respiratory control values is observed. The ADP/O ratios also indicate that oxidative phosphorylation is less efficient in these mitochondria.

Electron transport in mitochondria from fresh slices is fully inhibited by a low concentration (400 μM) of KCN (Fig. 1A). In contrast, mitochondria from aged tissue show a marked resistance ($\simeq$ 40%) toward the same concentration of cyanide (Fig. 1B). It should also be pointed out that the action of cyanide presents no lag phase and that the degree of inhibition does not increase with time. The fraction of electron transport that is resistant to cyanide can be fully inhibited by 1 mM SHAM (Fig. 1B).

The oxidation pattern of exogenous NADH is quite different from that of succinate (Fig. 1, C and D, Table I). Firstly, the rate of oxidation markedly increases during aging. Then, ADP/O ratios and RC values remain close to those of mitochondria from fresh slices. The ADP/O values for NADH are close to those of succinate. This indicates that the electrons given off by this substrate probably bypass the first site of phosphorylation, as is generally the case in plant mitochondria.

Cyanide not only totally inhibits electron transfer in mitochondria from fresh slices but also exerts a very strong inhibitory effect on mitochondria from aged slices. The subsequent addition of SHAM has no (Fig. 1C) or very little effect (Fig. 1D). In addition, when used as the first inhibitor, SHAM shows no inhibitory effect on the rate of oxidation under both conditions.

If malate is used as a substrate for mitochondrial oxidations, the same conclusions are reached as with succinate, the major difference lying in the relatively slow rates of oxidation of this substrate by potato mitochondria (*cf.* Table I).

From these results we conclude that aging induces new properties in the mitochondria of potato slices. An alternate, nonphosphorylative, cyanide-insensitive electron transfer pathway is brought into operation. The oxidation patterns of various substrates are modified. Changes in the rate of oxidation and a differential utilization of the two possible electron pathways are observed depending on the substrates which are oxidized.

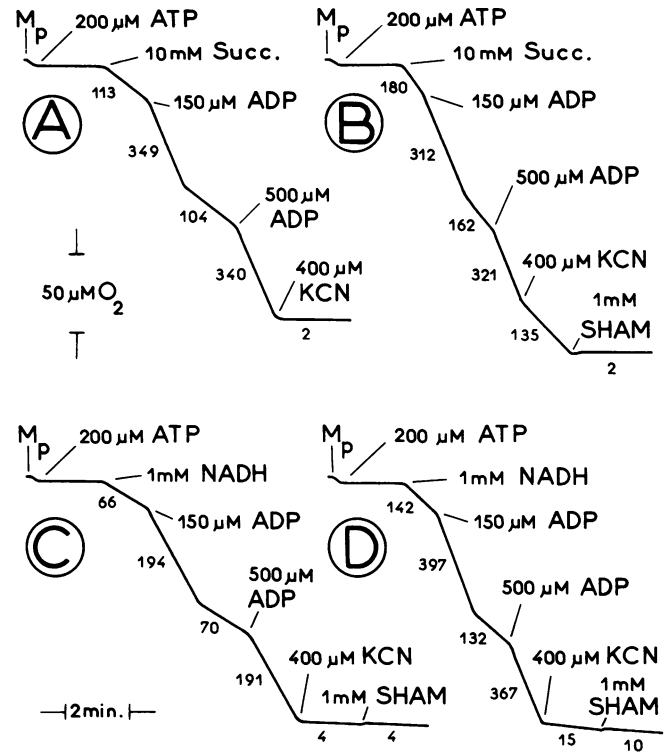

FIG. 1. Oxidation of succinate (Succ.) and NADH by mitochondria isolated from fresh or aged potato tissue slices. A and C: fresh tissue. B and D: aged tissue. Numbers along the traces indicate O_2 uptake in nmol/min·mg protein.

Effect of Chloramphenicol. When chloramphenicol is added

Table I. *Action of Chloramphenicol and Cycloheximide on Aging Process in Potato Tissue Slices*

The rates of oxygen uptake for the oxidation of various substrates by mitochondria isolated from fresh or aged tissue slices are expressed in nmol O_2/min·mg protein. The control for aged tissue was in the presence of 50 μg/ml of chloramphenicol (see text). Depending on the substrate used and the experimental condition, the figures are average values from 5 to 15 independent experiments.

		Mitochondria from fresh slices	Mitochondria from aged slices		
			Control	Chloramphenicol (350 μg/ml)	Cycloheximide (1 μg/ml)
State 3 rate	Succinate	328	310	311	236
	Malate	83	80	79	66
	NADH	234	346	332	163
State 4 rate	Succinate	100	158	155	76
	Malate	21	25	25	17
	NADH	85	123	116	60
R.C.	Succinate	3.28	1.96	2.00	3.10
	Malate	3.95	3.20	3.16	3.88
	NADH	2.75	2.81	2.86	2.72
ADP/O	Succinate	1.39	1.11	1.13	1.35
	Malate	2.33	2.12	2.06	2.28
	NADH	1.22	1.20	1.24	1.19

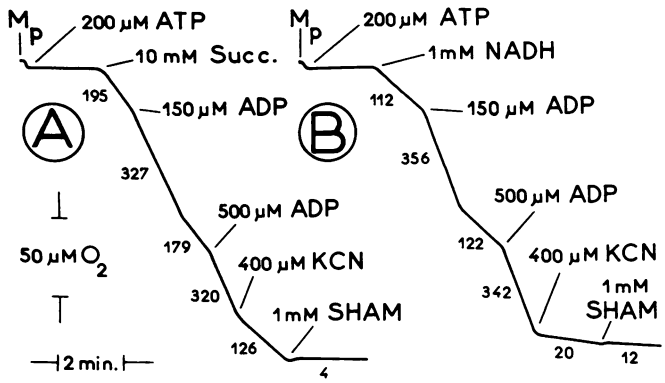

FIG. 2. Effect of the presence of chloramphenicol (350 μg/ml) during aging on the oxidation of succinate (Succ.) and NADH by mitochondria from potato slices. Numbers along the traces indicate O_2 uptake in nmol/min·mg protein.

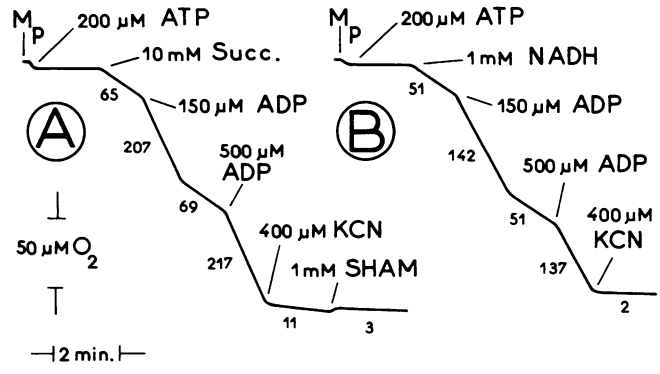

FIG. 3. Effect of the presence of cycloheximide (1 μg/ml) during aging on the oxidation of succinate (Succ.) and NADH by mitochondria from potato slices. Numbers along the traces indicate O_2 uptake in nmol/min·mg protein.

to the aging medium at a concentration of 350 μg/ml, the O_2 electrode traces for succinate and NADH oxidations (Fig. 2, A and B) are very similar to those obtained with mitochondria from normally aged tissue slices (Fig. 1, B and D). The oxidation rates and the efficiency of oxidative phosphorylation are nearly the same in both conditions (Table I). If the concentration of chloramphenicol is increased, no qualitative changes are observed. Only a slight reduction (10%) in the rate of oxidation of both substrates occurs, with no corresponding decrease in ADP/O ratios or RC values, when the chloramphenicol concentration in the aging medium is raised above 500 μg/ml.

These results show that chloramphenicol does not prevent the development of the alternate pathway or the rise in the rate of NADH oxidation. In addition, it does not change the qualitative and quantitative aspects of oxidative phosphorylation linked to these two substrates. The same conclusions are obtained with malate as substrate (Table I). These results also demonstrate that the low concentration of chloramphenicol (50 μg/ml), that is used to prevent bacterial contamination, is much too low to have any significant inhibitory effect on the aging process in potato tissue. On the other hand, it has also been checked by careful comparison of tissue slices aged in the absence or in the presence of 50 μg/ml of chloramphenicol that this concentration is also quite ineffective for inducing the development of the alternate pathway as in *Neurospora* (11, 16).

Effect of Cycloheximide. When aging takes place in the presence of 1 μg/ml of cycloheximide, the sensitivity of succinate oxidation to cyanide is almost as high as that observed in mitochondria isolated from fresh slices (Fig. 3A). The level of inhibition by cyanide is about 95%. The low residual electron transfer can then be completely abolished even by very low concentrations of SHAM. When added first, SHAM shows no effect, but the subsequent addition of KCN causes nearly 100% inhibition. Oxidative phosphorylation presents the same efficiency as in normally aged tissue, but it should be noted that both the state 3 and 4 rates of oxidation are slightly depressed by comparison with those of mitochondria isolated from fresh or normally aged tissue slices (Fig. 1, A and B).

Furthermore, in the presence of cycloheximide, one observes no rise in the intensity of NADH oxidation (Fig. 3B, Table I). The efficiency of oxidative phosphorylation is the same as in mitochondria from fresh or normally aged tissues. With NADH as substrate, the sensitivity to cyanide is almost 100%, in contrast with succinate oxidation for which a very low resistance (5%) can be observed. However, it should be pointed out that the rate of oxidation of NADH not only fails to increase as during normal aging, but even decreases below the rate found in mitochondria from fresh tissue, as does succinate oxidation (Table I).

The fact that cycloheximide depresses the rate of oxidation of these substrates is not due to an action *per se* of this inhibitor on mitochondrial oxidations, since we observed that cycloheximide had no effect on the oxidation rates or on the ADP/O and RC values when added to fresh potato slice mitochondria oxidizing various substrates.

From these results and similar ones obtained with malate (Table I), we conclude that cycloheximide prevents the development of the cyanide-resistant electron transport pathway as well as the enhancement of NADH oxidation, both of which normally occur during aging.

DISCUSSION

Besides the well known rise in respiration which takes place during the aging of potato tuber slices (14, 26), the above results show that a number of mitochondrial properties also undergo deep changes. The most spectacular one is the development of an alternate, cyanide-insensitive electron transport pathway which provides a second path for the electrons to reach O_2. The significance of this pathway is still obscure but bears upon the general problem of cyanide insensitivity in plant tissues. This pathway is known to be nonphosphorylative and to diverge from the main respiratory chain at the level of flavoproteins (3, 25).

One major current problem is to determine how much of this new pathway is used by the electrons flowing from substrates to O_2 (2). In aged potato slices, it seems that the possibility exists for the electrons from succinate or malate to reach O_2 through both pathways as evidenced by the decrease in the efficiency of oxidative phosphorylation, although there is no increase of the oxidation rates of these substrates during aging. On the other hand, the oxidation of NADH increases markedly, but the electrons from this substrate seem to move toward O_2 through the normal respiratory chain exclusively. As a rule, in cyanide-insensitive mitochondria, such as those from *Arum* spadix (6, 19), skunk cabbage (2, 25) or *Neurospora* (*poky* strain) (16), the electrons from succinate or NADH can flow to O_2 through both pathways. Nevertheless it must be recalled that the same difference as in aged potato slices between the oxidation patterns of NADH and succinate has also been observed in sweet potato mitochondria by Tomlinson and Moreland (27).

The development of the alternate pathway as well as the increase in the rate of NADH oxidation appear to be dependent on the synthesis of new proteins, which presumably are components of these two oxidative processes. Concerning NADH oxidation, Hackett *et al.* (14) have reported a severalfold increase in the NADH-Cyt *c* reductase activity (without significant increase in Cyt oxidase activity). This can explain the increase in the rate of NADH oxidation, but not the fact that electron

transfer from this substrate is most exclusively linked to the Cyt respiratory chain, with no possibility to diverge to the alternate pathway. A possible explanation could be that the increase in the rate of NADH oxidation does not involve the NADH dehydrogenase of complex I but rather the external NADH dehydrogenase located on the outer side of the inner membrane of plant mitochondria (10).

The situation appears to be more clearly defined in the case of the alternate pathway. In mitochondria from aged potato slices (8), one observes no increase in the amounts of Cyt belonging to the normal respiratory chain or in ferrosulfoproteins, which are thought to be the typical components of the alternate pathway. This observation is in accordance with the fact that there is no difference in the amounts of these components between mitochondria from cyanide-sensitive or cyanide-insensitive tissues either (9). Since it is well known that protein synthesis does occur during aging (7, 15), it appears that if the mechanisms of protein synthesis exert some control on these phenomena it must be at another level than that of the synthesis of cytochromes or ferrosulfoproteins.

The actions of two specific inhibitors of protein synthesis, chloramphenicol and cycloheximide, clearly show that only the cytoplasmic protein-synthesizing system has an effect on the changes in mitochondrial properties during aging.

The specificity of these inhibitors, however, can be questioned. Although there are some reports of inhibitory actions of chloramphenicol on energy-linked processes in mitochondria (24) and in plant tissues (18), our results show that the inhibitor does not decrease the rates of oxidation or the efficiency of oxidative phosphorylation even at a high concentration. On the other hand, in potato slices, chloramphenicol seems to have no positive effect on the development or on the activity of the alternate pathway, as is the case for some microorganisms such as *Neurospora* (11, 16). In this particular instance, however, it must be pointed out that higher concentrations of chloramphenicol (2 mg/ml) are required.

As to cycloheximide, some direct positive effects on the respiratory rate of storage tissue slices have been reported, implying some uncoupling of energy transduction process (12). However, these results are questionable according to some authors. In some tissues, evidence has been recently produced that cycloheximide primarily acts on the protein-synthesizing system with only very little or no action on energy flow in the cell (1, 17). From this and from our own observations, one can reasonably think that the decrease in oxidative capacities in mitochondria from tissues aged in the presence of cycloheximide is not due to an inhibitory effect of cycloheximide on these activities but rather to a direct effect on the mechanisms of protein synthesis.

It thus appears that the changes in mitochondrial activities associated with the aging process are dependent upon one or several proteins synthesized in the cytoplasm. In the case of enhanced NADH oxidation, this could be the NADH dehydrogenase system itself, as suggested by Hackett *et al.* (14), or it could also be a factor acting between the dehydrogenase itself and Cyt *b*, whose role would be to strengthen the link between these two components of the respiratory chain, so that the electrons from NADH become unable to use the alternate pathway.

As to the alternate pathway, the problem seems to be more complex. The scheme proposed by Edwards *et al.* (11) from their studies on *Neurospora* cannot apply to tissue slices. The hypothesis of a mitochondrially synthesized repressor acting on nuclear genes coding for the alternate oxidase cannot fit our data, since the addition of chloramphenicol to aging tissue slices has no stimulating effect on the development or on the activity of the alternate pathway.

Recently, Solomos and Laties (22, 23) have proposed another mechanism for the development of cyanide resistance in potato slices. According to their views, the alternate pathway is normally present in intact potato tubers since tuber respiration is not only cyanide-insensitive but is even stimulated by this inhibitor. Slicing of the tissue causes drastic alterations in membrane structure and composition, particularly at the phospholipid level. These alterations would be sufficient to impair the functioning of the alternate pathway. Upon aging of the slices, repair of the mitochondrial membranes, by synthesis of new molecules, would restore the alternate pathway. Whatever the true mechanism, according to this scheme the repair of the alternate pathway is also dependent on the synthesis of proteins which are either incorporated into the mitochondrial membrane, as regular membrane components, or are necessary for the synthesis of some membrane components, presumably phospholipids. In this respect, our results show that the cytoplasmic system for protein synthesis is implicated in the synthesis of these molecules. If we admit that all plant mitochondria possess the alternate pathway, at least in a latent state, these molecules could be the "link" between the two branched pathways. This link could be rather fragile, if we follow the views of Solomos and Laties (22, 23), but nevertheless absolutely necessary for the expression of cyanide insensitivity in plant mitochondria.

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LITERATURE CITED

1. Ap Rees, T. and J. A. Bryant. 1971. Effects of cycloheximide on protein synthesis and respiration in disks of carrot storage tissue. Phytochemistry 10: 1183–1190.
2. Bahr, J. T. and W. D. Bonner. 1973. Cyanide-insensitive respiration. I. The steady states of skunk cabbage spadix and bean hypocotyl mitochondria. J. Biol. Chem. 248: 3441–3445.
3. Bendall, D. S. and W. D. Bonner. 1971. Cyanide-insensitive respiration in plant mitochondria. Plant Physiol. 47: 236–245.
4. Bonner, W. D. 1967. A general method for the preparation of plant mitochondria. Methods Enzymol. 10: 126–133.
5. Chance, B. and G. R. Williams. 1956. The respiratory chain and oxidative phosphorylation. Adv. Enzymol. 17: 65–134.
6. Chauveau, M. 1976. La Chaîne respiratoire des mitochondries d'*Arum*. II. Inhibition de la voie d'oxydation insensible au cyanure. Physiol. Vég. 14: 325–337.
7. Click, R. E. and D. P. Hackett. 1963. The role of protein and nucleic acid synthesis in the development of respiration in potato tuber slices. Proc. Nat. Acad. Sci. U. S. A. 50: 243–250.
8. Dizengremel, P. 1975. La voie d'oxydation insensible au cyanure dans les mitochondries de tranches de Pomme de terre (*Solanum tuberosum* L.) maintenues en survie. Physiol. Vég. 13: 39–54.
9. Dizengremel, P., M. Chauveau, and C. Lance. 1973. Résistance au cyanure et teneur en ferrosulfoprotéines des mitochondries végétales. C. R. Seances Acad. Sci. Ser. D. 277: 239–242.
10. Douce, R., C. A. Mannella, and W. D. Bonner. 1973. The external NADH dehydrogenases of intact plant mitochondria. Biochim. Biophys. Acta 292: 105–116.
11. Edwards, D. L., E. Rosenberg, and P. A. Maroney. 1974. Induction of cyanide-insensitive respiration in *Neurospora crassa*. J. Biol. Chem. 249: 3551–3556.
12. Ellis, R. J. and I. R. MacDonald. 1970. Specificity of cycloheximide in higher plant systems. Plant Physiol. 46: 227–232.
13. Estabrook, R. W. 1967. Mitochondrial respiratory control and the polarographic measurement of ADP:O ratios. Methods Enzymol. 10: 41–47.
14. Hackett, D. P., D. W. Haas, S. K. Griffiths, and D. J. Niederpruem. 1960. Studies on development of cyanide-resistant respiration in potato tuber slices. Plant Physiol. 35: 8–19.
15. Kahl, G. 1974. Metabolism in plant storage tissue slices. Bot. Rev. 40: 263–314.
16. Lambowitz, A. M., E. W. Smith, and C. W. Slayman. 1972. Oxidative phosphorylation in *Neurospora* mitochondria. Studies on wild type, *poky*, and chloramphenicol-induced wild type. J. Biol. Chem. 247: 4859–4865.
17. Laüchli, A., U. Lüttge, and M. G. Pitman. 1973. Ion uptake and transport through barley seedlings: differential effect of cycloheximide. Z. Naturforsch. 28c: 431–434.
18. MacDonald, I. R., J. S. D. Bacon, D. Vaughan, and R. J. Ellis. 1966. The relation between ion absorption and protein synthesis in beet disks. J. Exp. Bot. 17: 822–837.
19. Passam, H. C. and J. M. Palmer. 1972. Electron transport and oxidative phosphorylation in *Arum* spadix mitochondria. J. Exp. Bot. 23: 366–374.
20. Schatz, G. and T. L. Mason. 1974. The biosynthesis of mitochondrial proteins. Annu. Rev. Biochem. 43: 51–87.
21. Schonbaum, G. R., W. D. Bonner, B. T. Storey, and J. T. Bahr. 1971. Specific inhibition of the cyanide-insensitive respiratory pathway in plant mitochondria by hydroxamic acids. Plant Physiol. 47: 124–128.
22. Solomos, T. and G. G. Laties. 1974. Similarities between the actions of ethylene and cyanide in initiating the climacteric and ripening of avocados. Plant Physiol. 54: 506–511.
23. Solomos, T. and G. G. Laties. 1975. The mechanisms of ethylene and cyanide action in

triggering the rise in respiration in potato tubers. Plant Physiol. 55: 73-78.
24. Stoner, C. D., T. K. Hodges, and J. B. Hanson. 1964. Chloramphenicol as an inhibitor of energy-linked processes in maize mitochondria. Nature 203: 258-261.
25. Storey, B. T. and J. T. Bahr. 1969. The respiratory chain of plant mitochondria. I. Electron transport between succinate and oxygen in skunk cabbage mitochondria. Plant Physiol. 44: 115-125.
26. Thimann, K. V., C. S. Yocum, and D. P. Hackett. 1954. Terminal oxidases and growth in plant tissues. III. Terminal oxidation in potato tuber tissue. Arch. Biochem. Biophys. 53: 239-257.
27. Tomlinson, P. F. and D. E. Moreland. 1975. Cyanide-resistant respiration of sweet potato mitochondria. Plant Physiol. 55: 365-369.
28. Van Steveninck, R. F. M. 1975. The "washing" or "aging" phenomenon in plant tissues. Annu. Rev. Plant Physiol. 26: 237-258.
29. Wiskich, J. T. and W. D. Bonner. 1963. Preparation and properties of sweet potato mitochondria. Plant Physiol. 38: 594-604.