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"Survival" of Mitochondria *in Vitro*

PHYSICAL AND ENERGY PARAMETERS

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ABSTRACT

Isolated mitochondria have been maintained active and coupled for 72 hours at 25 C. Survival (retention of respiratory control) is a function of incubation temperature and dependent upon aeration and substrate. ATP does not entirely substitute for substrate, indicating a need for products of active metabolism other than energy. An improvement in respiratory control is often observed during the first several hours of incubation. Sedimentation and resuspension at 24-hour intervals prolonged survival. As revealed by electron microscopy, mitochondria maintained their basic structure during a 72-hour period at 25 C.

Survival is a dynamic, energy-requiring process and must be distinguished from so-called "aging" of organelles at ice temperatures. As a manifestation of partial autonomy, survival may prove useful in assessing aspects of mitochondrial function and the mitochondrial-cellular interrelationship.

The prospects of prolonged incubation of isolated organelles were heightened by the reports of Ridley and Leech (7) and Giles and Sarafis (2) demonstrating the survival of isolated chloroplasts for several days. In earlier experiments (8) we have shown that mitochondria will retain respiratory control for 20 hr at 25 C when supplied with substrate and appropriate cofactors. The present paper describes the influence of physical and energy parameters that further extend the retention of respiratory control, referred to hereafter as survival, to at least 72 hr at 25 C. A preliminary report of this work has appeared (9).

MATERIALS AND METHODS

Avocados (*Persea americana* "Fuerte" Mill) were obtained from orchards in southern California or from local wholesale markets and used as a source of mitochondria.

Isolation Techniques. The isolation procedure used throughout these experiments was essentially that described by Lance *et al.* (3) with minor modifications. Peeled and pitted pieces of avocado were grated into isolation medium (1:3, w/v) composed of 0.25 M sucrose, 0.05 M potassium phosphate buffer (pH 7.2), 5 mM EDTA, 0.2% polyvinylpyrollidone (40,000 mol wt), 0.1% bovine serum albumin, and 5 mM β-mercaptoethanol. Grating was done below the liquid surface (10). Cellular debris was removed by centrifugation at 1,200g for 10 min. After additional centrifugation at 14,000g for 15 min the mitochondrial pellet was resuspended in "wash" medium (0.25 M sucrose, 0.05 M phosphate buffer (pH 7.2), 0.1% bovine serum albumin, and 5 mM β-mercaptoethanol). The suspension was centrifuged at 1,000g for 5 min and the resultant supernatant fraction at 8,000g for 10 min. The final pellet was resuspended in a few tenths of a milliliter of wash medium and held at 0 C, for a period seldom exceeding 1 hr, until the mitochondria were assayed or incubated at 25 C.

Incubation and Assay. As a *standard* incubation procedure based on earlier experiments (8), the mitochondria were suspended in 3 ml of reagent mixture (see legend, Fig. 1) contained in a 20-ml beaker. At the start of the incubation 0.4 μmole of ADP was also added, and the beaker was covered with parafilm and then placed in a shaking water bath at 25 C. To assay for oxygen consumption, the incubated mixture was transferred to a closed vessel equipped with a polarographic oxygen electrode. As the objective of this work was to test additional incubation parameters, the latter varied as described in each experiment. However, in all instances the incubation conditions were normalized (temperature adjusted, substrate added, etc.) just before the polarographic assay that was always conducted under the conditions described in the legend of Figure 1.

Respiratory control was expressed in the manner described by Chance and Williams (1). Specific activities were based on Lowry protein assays according to a modification by Miller (4).

In preparation for embedding and electron microscopy mitochondria suspended in incubation medium were transferred into a test tube containing 1/10 volume of 25% glutaraldehyde and placed at 0 C for 2 to 5 hr. After centrifugation at 10,000g for 10 min the pellet was rinsed and then resuspended in 2 ml of 2.5% glutaraldehyde in 0.1 M phosphate, pH 7.2. The fixed mitochondria were stained with 1% OsO_4 (in 0.1 M phosphate, pH 7.2) for 1.5 hr at 0 C and centrifuged, and the pellet was dehydrated with increasing concentrations of alcohol.

RESULTS

Survival Capacity of Various Intracellular Fractions. As an expedient in studying the effect of incubation conditions on survival, commonly used isolation procedures (3, 10) were adopted. One exception was the selection of centrifugation forces to obtain the final mitochondrial pellet. A comparison of organelle fractions obtained at increasing centrifugal forces indicated (Table I) that the organelle fraction sedimented at 1000g or at forces above 8000g had a low respiratory activity and little or no RC.[1] On the basis of these and other corroborative data the fraction sedimenting between 1000 and 8000g

[1] Abbreviations: RC: respiratory control; RCR: respiratory control ratio.

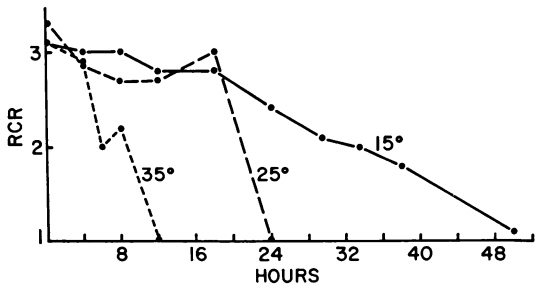

FIG. 1. Maintenance of mitochondrial respiratory control as a function of incubation time at 25 C. Mitochondria were incubated and assayed for oxygen consumption in the same reaction medium. The medium consisted of: 0.75 mmole of sucrose, 200 μmoles of P_i (pH 7.2), 30 μmoles of α-ketoglutarate, 0.3 μmole of NAD, 0.1 μmole of thiamine pyrophosphate, 0.4 μmole of ADP, 3 μmoles of $MgCl_2$, 0.01 μmole of CoA, 3 mg of bovine serum albumin, and 150 μg of chloramphenicol. Final volume: 3 ml + 0.02 to 0.03 ml with each subsequent addition of ADP. Assay temperature: 25 C.

Table I. *Survival Potential of Different Mitochondrial Fractions*

Each fraction obtained by centrifuging a once-washed mitochondrial suspension at increasingly higher forces. The resultant pellets were resuspended and used in standard assays.

Time at 25 C	Respiratory Control Ratio and Rate of O_2 Uptake				
	1,000*g*	4,000*g*	8,000*g*	12,000*g*	18,000*g*
hr					
0	3.6[1] (43)[2]	3.4 (54)	3.4 (79)	1 (26)	1 (21)
2	4.4 (43)	3.4 (59)	5.0 (96)	1 (19)	...
8	2.8 (32)	2.0 (39)	3.2 (74)	...	...
18	1.4 (14)	2.5 (19)	...	...	...

[1] Respiratory control ratios.
[2] Number in parentheses = nmoles O_2/min·mg protein.

was chosen as having the maximal survival potential and was used in all subsequent experiments.

Effects of Incubation Temperature. Mitochondria were incubated at 15, 25, and 35 C for specified lengths of time before being assayed for respiratory activity at 25 C. It was clear from the results obtained (Fig. 1) that maintenance of RC is highly dependent upon incubation temperature. It would appear that accelerated metabolic activity shortens survival; however, as shown below, constraints on metabolism will also limit survival. Since our intention was to investigate survival as a dynamic function and not as a passive state, it seemed logical to examine the nature of survival at a temperature that permitted active metabolism. In all subsequent experiments 25 C was used as a fixed incubation (survival) temperature.

Survival and Mitochondrial Concentration. It has been demonstrated (6, 12) that the concentration of mitochondia in suspension will affect their metabolic performance. The results of an experiment to study this parameter with respect to survival *in vitro* are shown in Figure 2. Each 3 ml of incubation medium contained either 0.43 mg or twice or four times that amount of mitochondrial protein. In general, the respiratory control ratio (left graph) remained at a slightly higher level in the most dilute mitochondrial suspension.

Romani and Monadjem (8) had noted previously that many mitochondrial preparations, especially those with high initial RC, experienced a further enhancement of RC during the first few hours of incubation. This trend (seen in Figs. 2, 4, 5, 6 and Table I) is of particular interest as it implies a positive response to the *in vitro* condition. Since the time-dependent increase in both RC and rate of oxygen consumption (Fig. 2) was suppressed at higher mitochondrial concentrations, all subsequent incubations were held within the range of 0.5 to 1 mg of mitochondrial protein per ml.

Effects of Aeration. We have previously reported (8) that, following a period of incubation at 25 C, initial polarographic readings could be directly related to the metabolic rate of the organelles. As the mitochondria became less active, the initial postincubation O_2 readings were higher, indicating that an equilibrium was maintained between rates of oxygen consumption and air diffusion into the incubation medium. The results of an experiment in which rates of aeration were controlled by varying the reciprocating rate of the shaking water bath (Lab.-Lime Instr., Melrose Park, Ill.) are seen in Figure 3. With no shaking at all during incubation RC declined for the first 4 hr and then stabilized at a value between 2 and 3. With moderate shaking (50 strokes/min) RC was maintained at its highest level. Greater aeration (150 strokes/min) resulted in uncoupling and a total loss of RC in the interval between 12 and 18 hr of incubation. On the basis of these and similar data obtained in other experiments, *50 strokes/min* was used as a standard shaking speed in all subsequent studies.

Effects of Reduced Oxygen Tension. To substantiate an oxygen dependency for survival, mitochondria were incubated under standard conditions (25 C, 50 strokes/min) in beakers exposed to air or N_2. Prior to polarographic assay each beaker was shaken an additional 5 min in the presence of air. Results of this experiment are shown in Figure 4. Although N_2 (air in the control) was bubbled through the incubation medium prior

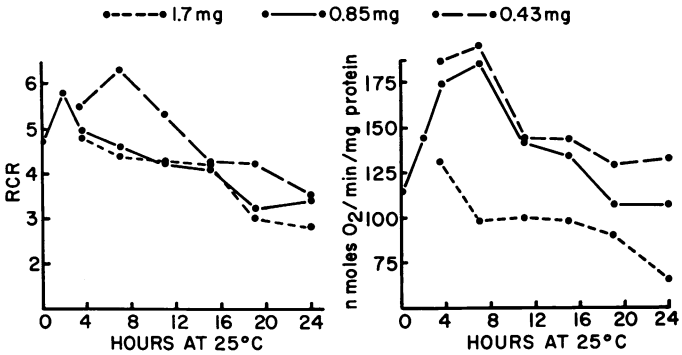

FIG. 2. Maintenance of mitochondrial respiratory control (left graph) and oxidative capacity (right graph) as a function of organelle concentration in the incubation medium. Shown are the state 3 oxidative rates and the respiratory control ratios.

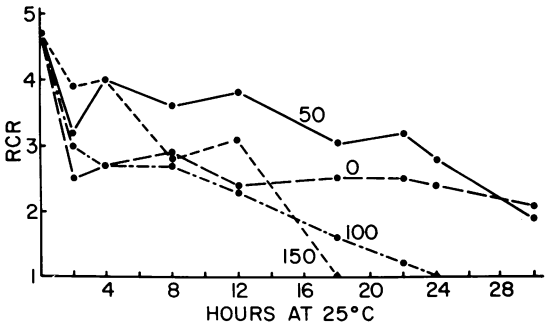

FIG. 3. Maintenance of mitochondrial respiratory control as affected by aeration. Numbers associated with each line refer to the reciprocations per minute (shaking speed) of the water-bath shaker.

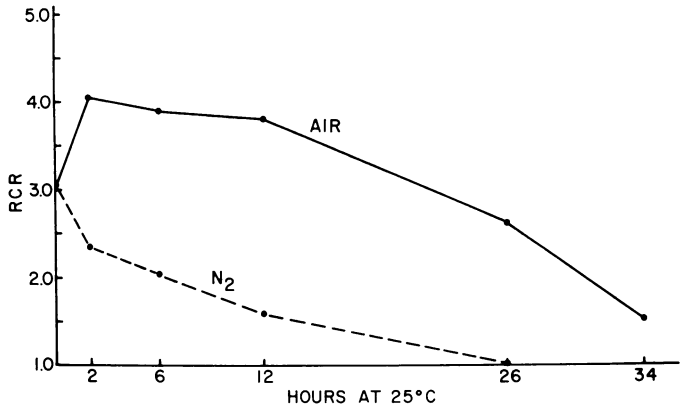

FIG. 4. Effects of lowered oxygen tensions on the survival of isolated mitochondria. The incubation were in equilibrium with air or nitrogen. Before polarographic assay air was reintroduced into the quasi-anaerobic suspensions by shaking under normal (air) conditions for 5 min.

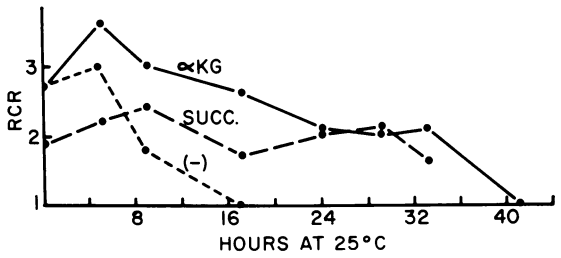

FIG. 5. Substrate requirement for mitochondrial survival. Each incubation vessel contained either 30 μmoles of α-ketoglutarate (αkg), 30 μmoles of succinate (Succ.), or no substrate (—). Assay at the end of the incubation period was performed in the presence of the original substrate and after the addition of 30 μmoles of α-ketoglutarate to the "no substrate" medium.

to addition of the mitochondria, approximately 10% of the normal O_2 at partial pressure remained in the mixture at the start of the experiment. Some air intrusion would also have occurred as the gassing hood was lifted to remove each successive incubation beaker. Nonetheless, survival was definitely limited under the quasi-anaerobic conditions. Optimal O_2 tensions would obviously be a complex function of temperature, rate of aeration, and mitochondrial concentration; but the need for oxygen and metabolic activity for the maintenance of RC is clear.

Substrate Requirement. A direct assessment of the dependency on active metabolism was obtained by omitting substrate during incubation. As shown in Figure 5, survival beyond the first 4 hr of incubation was seriously limited by the absence of substrate. Both succinate and α-ketoglutarate sustained mitochondrial RC. Citrate and pyruvate (not shown) were equally effective in sustaining survival. These data clearly indicate that a source of metabolic energy is required if the mitochondria are to remain coupled for an extended period of time.

Energy Requirement. Based on preliminary observations catalytic amounts of phosphate acceptor (0.4 μmole of ADP) were routinely included in each incubation mixture. Further tests (not shown) of this requirement confirmed that optimal levels of ADP were in the range of 0.4 to 4 μmoles; 20 μmoles of ADP per 3 ml reaction mixture were excessive.

A requirement for catalytic amounts of ADP again implies that metabolically derived energy is essential for survival. The question arises whether ATP can serve as an alternate energy source. As indicated in Figure 6, added ATP (5 μmoles/3 ml incubation medium) in the absence of substrate caused a significant improvement in RC during the first 12 hr. However, there was a decline thereafter, and no RC remained after 36 hr. Mitochondria incubated without substrate or ATP maintained a minimal level of respiratory control longer than those supplied with ATP alone. In this respect the experiment was somewhat anomalous, being one of the few instances where the lack of substrate did not result in a relatively rapid and total loss of RC.

The presence of both ATP and substrate in the reaction mixture resulted in a maximal level of sustained RC, including a gradual increase in RC up to about 30 hr at 25 C (Fig. 6). This difference in response to ATP, as conditioned by the absence or presence of substrate, was observed repeatedly. One can surmise that active metabolism and products therefrom, in addition to derived energy, are essential for extended mitochondrial survival.

That ATP was being utilized and converted to ADP (or AMP) was evidenced by the time course of O_2 consumption following the addition of α-ketoglutarate to the mitochondria surviving without substrate. Energy liberated from ATP should result in an accumulation of ADP and hence a prolonged state 3 upon the subsequent addition of substrate. This was experimentally confirmed. In Figure 7 the oxygen electrode trace observed at 0 hr of incubation may be compared with those observed after 29 hr of incubation in the

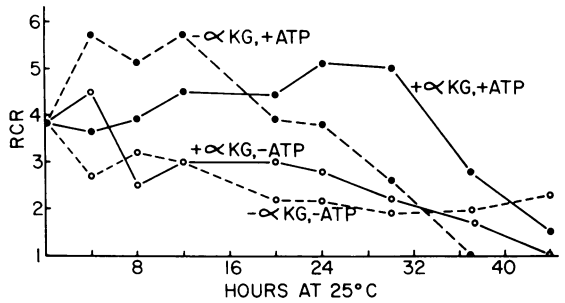

FIG. 6. Effects of different combinations of substrate (α-ketoglutarate) and energy source (5.0 μmoles of ATP) on the survival of mitochondria *in vitro*.

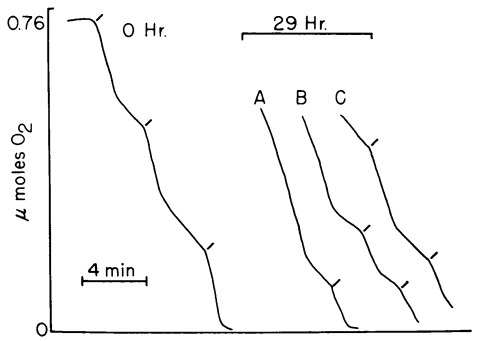

FIG. 7. Evidence for the accumulation of ADP when mitochondria are incubated in a complete reaction medium less substrate but plus 5 μmoles of ATP. First (0 hr) trace indicates the respiratory response to ADP of freshly isolated mitochondria. Traces A, B, and C depict the oxidative response of similar mitochondria to the addition of α-ketoglutarate after 29 hr of incubation at 25 C in the absence of substrate. A: Assay began immediately after addition of substrate; B: 2 min; and C: 8 min after addition of substrate. Short slant lines indicate the addition of ADP.

Table II. *Survival of Mitochondria Incubated in Standard or in Bulk*

Time at 25 C	Respiratory Control Ratio and (ADP/O)[1]	
	Standard incubation	Standard incubation after 24-hr incubation in bulk
hr		
0	3.8 (2.6)	(bulk)[2]
2	4.0 (2.5)	...
6	3.6 (2.5)	...
12	3.3 (2.2)	...
24	2.6 (2.1)	4.4 (2.6)
32	1.7 (1.5)	3.3 (2.0)
44	No RC (0)	2.2 (1.5)

[1] Based on an estimated 0.76 μmole of O_2 per 3.2 ml of air-saturated medium.

[2] The medium as described in the legend to Figure 1 was used in both "standard" and "bulk" incubations. Physical aspects of the incubation procedures differed as described in the test.

absence of α-ketoglutarate. Mitochondria and reaction mixture were transferred to the polarographic assay cuvette either immediately after the addition of substrate (A), 2 min after addition of substrate (B), or after approximately 8 min (C). Sufficient ADP or other phosphate acceptor had accumulated during the preceding 29 hr to sustain state 3 respiration for a period of 2 or 3 min (A, B). However, at a point between 2 and 8 min after the addition of substrate the previously accumulated phosphate acceptor had now been phosphorylated, resulting in state 4 respiration (C).

The Effects of Bulk Incubation and of Resuspension on Mitochondrial Survival. As an alternate to the standard incubation a "bulk" method was tried wherein 10 times the usual amount of mitochondria were placed in 12 ml of reaction mixture (mitochondria:reaction mixture = 2½ times that in the standard incubation) contained in a 100-ml beaker. After 24 hr at 25 C the bulk mitochondrial suspension was chilled for 1 hr and then centrifuged at 8000*g* for 10 min. The mitochondrial pellet was resuspended and apportioned in 20-ml beakers with fresh reaction medium in "standard" fashion. A comparison of the two incubation procedures with regard to their effects on mitochondrial survival is noted in Table II. RC, and associated ADP/0, were maintained for about 32 hr during the standard incubation. However, after 24 hr of bulk incubation the pelleted and resuspended mitochondria exhibited an RCR actually slightly higher than the 0 hr value and a subsequent survival pattern paralleling that observed 24 hr earlier. It appeared that either chilling, bulk conditions *per se*, pelleting and resuspension, or reincubation in fresh reaction medium had nearly doubled the period of survival. Each of these possibilities was examined.

In experiments not shown appropriate aliquots were taken at various times directly from bulk-incubated mitochondria, diluted to 3 ml with fresh incubation medium, and assayed for RC, thus bypassing the chilling, centrifugation, and resuspension steps. Little increase in survival was discerned, indicating that bulk incubations *per se* had minimal effects on survival.

To test the remaining possibilities noted above, mitochondria were incubated in standard fashion (20-ml beakers) with or without 1 hr of prechilling before polarographic assay. The remainder of the same mitochondrial preparation was incubated in two bulk suspensions. At four intervals—20, 44, 56, and 81 hr—mitochondria in the two bulk suspensions were chilled, pelleted, resuspended, and bulk-incubated once again, one always in fresh medium and the other always in its original or old medium. A small portion of the resuspended mitochondria was used immediately for polarographic assay.

The results of this experiment are summarized in Table III. One hour of prechilling had minimal effects on survival, and mitochondria incubated under standard conditions had lost all RC by 44 hr. In contrast, mitochondria incubated in bulk and periodically centrifuged and resuspended maintained RC after 44 and 56 hr at 25 C. When centrifuged and resuspended after 81 hr at 25 C the organelles had lost all control and most of their respiratory activity. It would seem, therefore, that the processes of pelleting and resuspension, normally thought to stress the mitochondria, actually contributed to their extended survival. Why this should be the case is not at all clear at this time.

Significant in this experiment (Table III) was the fact that resuspension in fresh as opposed to the same (old) reagent medium caused very little, if any, enhancement in survival of the organelles. Ultimate inactivation and death cannot, therefore, be attributed to the accumulation of toxic substances. An actual lowering of total oxidative activity (values in parentheses, Table III) following resuspension in new medium indicated that some diffusable component essential to respiratory activity may have been lost or diluted.

Based on the above findings maximal survival of mitochondria, with respect to both respiratory activity (Fig. 8, upper graph) and respiratory control (lower graph), was obtained when bulk incubation was combined with periodic centrifugation and resuspension. Under these conditions mitochondria have retained an RCR of about 3 for 72 hr.

DISCUSSION

The above experiments permit the following conclusions. (*a*) Mitochondria can survive at 25 C for periods of time measurable in days. (*b*) Survival is affected by a variety of physical parameters and may well be influenced by numerous

Table III. *Mitochondrial Survival as Affected by Chilling and Resuspension in Old versus Fresh Medium*

Before polarographic assay mitochondria incubated in bulk were chilled for 1 hr, pelleted by centrifugation for 10 min at 8000 × *g*, and resuspended either in the original or in fresh incubation medium.

Time at 25 C	Standard Incubation[1]		Bulk Incubation[1]	
	No prechilling	Prechilled 1 hr	Old medium	New medium
hr				
0	3.5[2]	...	...	...
8	3.2	...	...	...
20	2.8	2.5	4.0 (103)[3]	3.9 (72)
25	2.7	2.4	...	...
32	1.4	1.9	...	...
44	1.0	1.0	2.4 (64)	2.2 (52)
56	...	...	1.9 (69)	2.2 (29)
81	...	...	1.0 (15)	1.0 (24)

[1] Standard and bulk incubations were both composed of complete medium (see legend, Fig. 1) but differed only in total volume and in ratio of mitochondria to medium as described in the text.

[2] Respiratory control ratios.

[3] Number in parentheses = nmoles O_2/min·mg mitochondrial protein.

untested variables operative during isolation, incubation, and polarographic assay of the mitochondria. (*c*) The functional capability of mitochondria, with respect to rate of metabolic reactions and respiratory control, is not only relatively stable but may often improve during the first several hours of incubation.

These observations have influenced our views with regard to so-called "aging" of mitochondria and the stability of the organelles as affected by rapidity of isolation. The holding of mitochondria at ice temperature is often referred to as "aging" with accompanying losses in function occurring after 2, 3, or more hours. Recognizing now that survival is an energy-demanding process, mitochondria may well be expected to age and deteriorate under conditions (0 C, lack of substrate, etc.) that preclude metabolism and derivation of needed energy. In this sense aging and deterioration are not a reflection of organelle weakness but, conversely, the result of constrictions that prevent an otherwise sound organelle from responding to the stress of time.

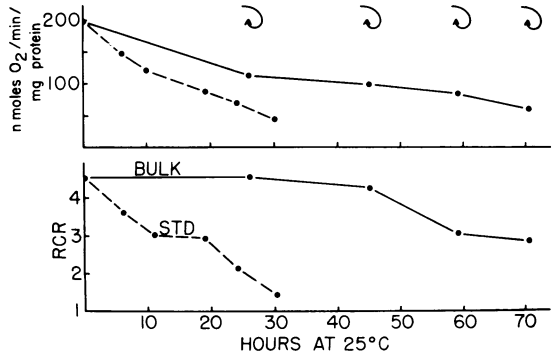

FIG. 8. The combined effects of bulk incubation and repelleting on the survival of isolated mitochondria. Each curled arrow represents a centrifugation and resuspension of the mitochondria. Upper graph: Oxidative rates in state 3; lower graph: respiratory control ratios. The ADP/0 values for the 0-, 26-, 45-, 59-, and 72-hr assays were 2.0, 2.1, 2.2, and 2.1, respectively.

Especially rapid methods of isolation have been shown to favor tightly coupled and stable (3 hr at 0 C) mitochondria (5, 6, 11). However, the maceration procedures employed with fruit tissues, designed to affect maximal control of pH (10), are particularly slow. One-half hour, and often longer, is required to macerate the several hundred grams of tissue normally used in our experiments. Perhaps a fortuitous combination of tissue characteristics, use of protective agents, and control of pH have resulted in long-lived organelles from avocados and pear fruit. Nonetheless, the results do indicate that the need for rapid isolation procedures is not a general posit for all mitochondria but a reflection of inadequate protective measures for a particular tissue, or set of conditions. Certainly, once free of vacuolar contaminants and supplied with substrate and essential cofactors, neither several hours at 25 C nor repeated pelleting and resuspension (Fig. 8) appear to harm the organelles.

Comparative studies of mitochondria have been fraught with uncertainties. Among these, as shown by Raison and Lyons (6), is the variable stability of mitochondria isolated from different tissues. In their studies mitochondria either declined in activity after 3 to 5 hr at 0 C or remained relatively stable, dependent upon tissue sources. It is now clear that under some conditions mitochondria may actually improve with time to further complicate comparative evaluations. However, it seems reasonable to expect that "surviving" mitochondria more nearly reflect the organelles' condition *in vivo* and should thus expand the prospects of examining mitochondrial status in relation to tissue source or physiological condition such as disease or other forms of stress.

Mitochondrial survival at this juncture may be equated to the attainment of coupled organelles over a decade ago. Many interrelated parameters affecting tissue source, isolation, incubation and assay must be assessed to achieve the optimal conditions. There is no evidence to indicate that 72 hr is an upper limit for mitochondrial survival.

The fact that pelleting at 8,000*g* and resuspension result in improved RC (Tables II and III, Fig. 8) negates the possibility that significant disintegration into active submitochon-

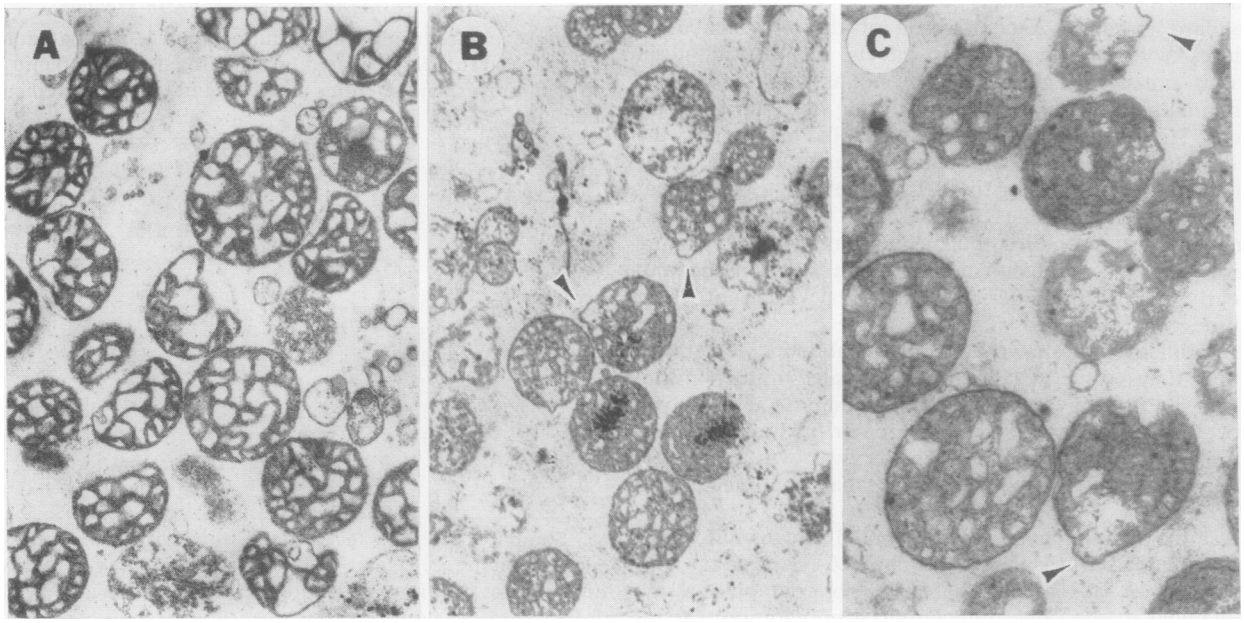

FIG. 9. Electron micrographs of avocado mitochondria fixed in glutaraldehyde after incubation for a few minutes (A) or 72 hr (B, C) at 25 C. A and B: × 18,000; C: × 24,000.

drial particles has occurred during incubation. In one established method submitochondrial particles are pelleted at 100,000*g* after discarding the *mitochondrial* fraction collected at 10,000*g* (13). We have also noted (Romani and Ozelkok, in preparation) that sucrose density centrifugation characteristics of fresh and 96-hr-old mitochondria are very nearly the same. Finally, electronmicrographs (Fig. 9) reveal that the mitochondria remain readily identifiable organelles after 72 hr at 25 C. Some swelling and outward distortions of the mitochondrial membrane were frequently observed after prolonged incubation. The meaning of this preliminary observation must be left to conjecture although there are some resemblances to the protuberances on the surviving chloroplasts examined by Ridley and Leech (7).

Bacterial contamination must be contended with in all extended incubations. Our use of chloramphenicol at 50 μg/ml adequately controlled bacterial contaminants for the first 20 hr; however, with extended survival periods even 100 μg of chloramphenicol per ml of medium did not afford bacterial control. Streptomycin and penicillin were also ineffective, and bacterial counts in the order of 10^8/ml were observed after 3 days of incubation. Although the inhibitors appeared to have minimal effects on miochondrial oxidations and RC, their use must surely inhibit protein synthesis, a likely essential to the further extension of survival. Our efforts to obtain *completely* sterile mitochondrial preparations have been unsuccessful, but a promising approach is indicated by the experiments of Giles and Sarafis (2) where the lysozymic component of hen egg albumin controlled bacterial contaminants in incubated chloroplast suspensions.

Finally, "survival" has been used by us to signify the retention of energy-linked functions and a reasonable degree of gross mitochondrial structure. Survival does have broad implications although not much more so than "autonomy"—the two are logically interlinked.

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