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THE PHYSICAL ENVIRONMENT AND OXIDATIVE AND PHOSPHORYLATIVE CAPACITIES OF HIGHER PLANT MITOCHONDRIA

GEORGE G. LATIES[1]

KERCKHOFF LABORATORIES OF THE BIOLOGICAL SCIENCES, CALIFORNIA INSTITUTE OF TECHNOLOGY, PASADENA, CALIFORNIA

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Introduction

The extensive and prolonged cytological interest in the mitochondria has been complemented in recent years by an interest in the biochemical and enzymological properties of these intracellular bodies. With the recognition that the greater part of the enzymes concerned with the aerobic respiratory metabolism were intimately associated with the cell particles (**5, 11, 14**), a good deal of attention was devoted to the relation of the structure of the mitochondria to their function.

The mitochondria were observed to be discrete osmotic systems (**5, 14, 29**), and their gross structure, at least, was shown to be related to the tonicity of the environment. Fundamental alterations in the biochemical properties of the cell particles were seen to follow upon osmotically evoked physical changes (**12, 13, 14**), with the consequence that the physical integrity of the mitochondria was considered to be a prerequisite for their coordinated biochemical activity (**8**). Although the concept of structural inviolability as a requirement for organized enzymatic activity has been revised to some extent as a result of the solubilization of certain mitochondrial enzymes (**9, 34, 37**), there is still every indication that the remarkable biochemical integration of the mitochondria is closely related to their precise physical organization.

The loss of certain enzymatic functions following exposure of cellular particles to a hypotonic environment (**12, 13, 14**) has in the past been attributed primarily to the spatial disorientation of components of the mitochondrial complex (**8**). More recently, however, additional consequences of hypotonic exposure have been recognized. The cofactors of oxidation and phosphorylation have been shown to be subject to degradation by enzymes contained both in the cytoplasm and in the mitochondria themselves (**19**). The tonicity of the environment, in turn, frequently affects the effective

[1] Present address: Department of Botany, University of Michigan, Ann Arbor, Michigan.

concentration or activity of such degradative enzymes (**18**). In addition to the recognized consequences of hypotonic exposure mentioned above, the studies which follow describe two situations in which hypotonicity is a necessary but insufficient condition to bring about profound changes in the enzymological characteristics of the mitochondria. Both the oxidative and phosphorylative capacities of the mitochondria may be adversely affected by a low ambient tonicity. Some effort will be made below to distinguish inactivation caused by hypotonicity *per se* from inactivation brought about by other factors, yet depending upon hypotonicity as a prerequisite. The salient question underlying such studies is whether or not the coupling of oxidation to phosphorylation is obligatory in the several oxidations under consideration.

The problems investigated herein are to some extent peculiar to plant tissues. For example, the succinoxidase system, one of the most readily demonstrable oxidase systems in homogenates of animal tissues, initially proved difficult to demonstrate in leaf breis (**4**), although all presumptive evidence (**20**) indicated the enzyme to be present in intact leaves. It seemed reasonable to assume that the intermixing of vacuolar contents with the cytoplasm was in part responsible for the difficulty, as well as the failure to observe particular precautions with respect to the tonicity of the environment. Some of the evidence offered below indicates these suppositions to be correct.

Particles from various plant tissues have previously been prepared (**2, 6, 28**) which have exhibited both succinoxidase and cytochrome oxidase activity. By and large, these two systems are able to survive considerable manipulation, and it appears probable that the preparations described in the past have been suspensions of mitochondrial fragments similar to those studied by KEILIN (**17**). Recent studies (**3, 21, 26**), however, have observed the preparative requirements for the isolation of relatively intact mitochondria (**14, 36**), with the result that these studies describe plant particles capable of a much broader variety of enzymatic activity than has hitherto been reported in plant preparations.

It is more than likely that the cytoplasmic particles prepared as described in the next section do not represent a physically homogeneous cellular fraction. In particular, the work of STAFFORD (**38**) indicates that centrifugal techniques as used herein may effect rather good separation of the biochemical systems found in a plant homogenate without simultaneously producing fractions which are completely uniform when viewed in the microscope. In this text, then, the term *mitochondria* will be used in the earliest sense, as described by BENDA [see NEWCOMER (**27**)], simply to designate small threads or small grains, and will be used interchangeably with the term *particles*. A diversity of mitochondrial sizes and shapes may be expected, especially in meristematic tissue (**10**). Although perhaps no precise morphological entity is obtained by the centrifugal method as used herein, the separation of enzymological and biochemical characteristics at least reflects the existence of different functional units within the cell.

Methods

Heads of cauliflower (*Brassica oleracea*) obtained at local markets were washed with tap water, rinsed with distilled water, blotted dry, wrapped in a damp towel, and cooled in the refrigerator. The topmost 2 to 3 mm. of the immature inflorescences (referred to as cauliflower buds in the text) was then removed with the aid of a plastic grater, and the remainder of the head discarded. Buds were homogenized in a chilled mortar with approximately seven parts of water or solution, and with one-third of the bud weight reagent-grade sea-sand. All solutions were kept ice-cold, and all manipulations, including centrifugation, were carried out in a cold room maintained at 2 to 4° C. Buds were homogenized to a paste with one quarter of the requisite solution, the remaining solution being added thereafter. The pH of water or sucrose homogenates was approximately 6.6.

Ordinarily, 12 gm. of chilled buds was homogenized with 80 ml. solution and centrifuged for 5 minutes at 500 × g. The supernatants were decanted into 50-ml. plastic centrifuge tubes through a single layer of muslin and centrifuged for 20 minutes at 20,000 × g. in a Servall SS-1 angle-head centrifuge. The supernatant was siphoned off with a water pump. The precipitate was resuspended in several ml. of ice-cold water or solution with a power-driven plastic pestle which fit loosely into the centrifuge tube. The volume was then brought to 25–30 ml., and this suspension centrifuged at 20,000 × g. for 20 minutes. The precipitate from the second high-speed centrifugation was suspended in 1.0 ml. of water or solution with the plastic pestle, and was then transferred to a test tube approximately 10 mm. in diameter. With the tube held in an ice bath, the suspension was gently homogenized for 10–15 seconds with a small snug-fitting power-driven glass pestle. The volume was finally brought to 1.5 to 2.0 ml., and 0.5 ml. of this material, containing approximately 0.5 mg. protein nitrogen, was used as enzyme source in the manometer flask. Following the final high-speed centrifugation, precipitates were frequently combined, the final volume being correspondingly adjusted. The centrifugation of the initial homogenate, cleared of debris, is referred to as the first washing or first suspension. Thus, the procedure described above entails two washings. Additional washings, when specified, were carried out in the same manner as the second washing.

In each experiment an aliquot (0.2 ml.) of the final enzyme suspension was diluted 10 times, following which duplicate samples (0.8 ml.) of the diluted suspension were transferred directly to Folin-Wu tubes for micro-Kjeldahl digestion with 1.0 ml. of digestion mixture (410 mg. mercuric acetate, 35 mg. selenium dioxide, 200 ml. concentrated H_2SO_4, brought to 1.0 l. with water). Two successive additions of 2 drops of 30% H_2O_2 were provided during the digestion. The digest was diluted, Nesslerized, and the color estimated with a Klett colorimeter.

Manometric determinations were by the direct method of Warburg, and were carried out at 30° C with KOH and filter paper in the center well. Reagents were added to the manometer flasks, and the flask contents chilled

in a tray of ice before addition of the enzyme. If required, Mg^{++} or fluoride were normally added after the other reagents and the enzyme (**32**). Readings were taken at 10-minute intervals, and the $Q_{O_2}(N)$ calculated on the basis of the first two 10 minute readings.

Phosphate determinations were carried out as described by ALLEN (**1**). At zero time and at suitable intervals thereafter aliquots of the reaction mixture were mixed with equal volumes of ice-cold 2 *N* HCl. Following low-speed centrifugation of this mixture, aliquots of the supernatant were taken for the estimation of phosphate.

Coenzyme I (65% purity) was obtained from Sigma Chemical Co., adenosinetriphosphate (98–100% purity) from the Pabst Brewing Co., and cytochrome C from the Nutritional Biochemicals Corp. Hexokinase prepared from yeast was kindly supplied by Dr. J. M. Campbell. This enzyme preparation, diluted 10 times, effected the phosphorylation of 10 μmoles glucose per minute per ml. enzyme. Abbreviations used in the text and legends are as follows: Diphosphopyridine nucleotide (Coenzyme I, or Co I); Adenosinetriphosphate (ATP); 2,4-dinitrophenol (DNP).

Experimental results

OSMOTIC INJURY IN THE PRESENCE OF SOLUBLE CELL COMPONENTS

Among the numerous oxidative systems associated with the mitochondria, the succinoxidase complex has been thought to be the least fragile. Whereas certain enzymatic functions of the mitochondria are lost upon exposure of the mitochondria to a hypotonic environment (**12, 14, 33**), the succinoxidase system has long been considered able to withstand such treatment (**17, 33**). However, in an earlier study (**22**) wherein the washing of relatively intact pieces of cauliflower bud tissue was carried out at room temperature, it was observed that the succinoxidase activity of particles prepared from washed tissue was considerably less than that of particles prepared from unwashed tissue. When tissue was washed in water at 1 to 2° C or at room temperature in hypertonic sucrose, mannitol or KCl, little inactivation of succinoxidase was subsequently perceived (**22**). These observations suggested that injury due to washing was in fact a consequence of the hypotonicity of the environment, but was not to be attributed to a simple physical process (swelling) *per se*. Experiments to test these hypotheses were therefore carried out with mitochondrial suspensions.

When tissue was homogenized at 0° C in water, sufficient sucrose added to one aliquot to give a final concentration of 0.5 M and the homogenates then incubated at room temperature, no lessening of the ensuing inactivation was observed in the presence of sucrose. However, when homogenization was carried out directly in either water or 0.5 M sucrose and the succinoxidase activity of the particles prepared from each homogenate was compared, the effect of sucrose in preventing inactivation was considerable (table I). This protection was manifest whether subsequent incubation at room temperature was carried out or not. That the effect of sucrose is attributable

TABLE I

THE EFFECT OF TONICITY DURING PREPARATION AND SUBSEQUENT SUSPENSION ON MITOCHONDRIAL ACTIVITY.

Expt.	Substrate	Preparative medium (first suspension)	Second Suspension	Sucrose molarity final suspension	Tonicity in flask	$Q_{O_2}(N)$
1	Succinate	Sucrose	Sucrose			362
		Sucrose	H_2O	0.5	High	362
		H_2O	Sucrose			228
		Sucrose	Sucrose			120
		Sucrose	H_2O	0.15	Low	162
		H_2O	Sucrose			166
2	α-Keto-glutarate	Sucrose	Sucrose			220
		Sucrose	H_2O	0.5	High	89
		H_2O	Sucrose			11
		Sucrose	Sucrose			62
		Sucrose	H_2O	0.15	Low	8
		H_2O	Sucrose			0
3	Malate	Sucrose	Sucrose			284
		Sucrose	H_2O	0.5	High	144
		H_2O	Sucrose			0
		Sucrose	Sucrose			95
		Sucrose	H_2O	0.15	Low	111
		H_2O	Sucrose			58

Final concentration of reagents in manometer flasks as follows: For succinate oxidation, substrate, 2×10^{-2} M; phosphate buffer (KH_2PO_4-Na_2HPO_4) pH 7.3, 2×10^{-2} M; cytochrome c, 2×10^{-5} M. When tonicity in flask is "Low," no additional sucrose added beyond that in which enzyme (0.5 ml.) is suspended. When tonicity in flask is "High," 0.6 ml. 1.5 M sucrose added. For α-ketoglutarate oxidation, ATP and $MgSO_4$ added to final concentrations of 10^{-3} M and 1.8×10^{-3} M, respectively. For malate oxidation: As for α-ketoglutarate, plus CoI, 3.3×10^{-4} M final concentration. Final volume brought to 3.0 ml. Sucrose 0.5 M where no concentration is specified. $Q_{O_2}(N)$ indicates $\mu l.O_2$ consumed per mg. protein nitrogen per hour.

to its role as an osmotic agent is clear from the data of table II and from figure 1, wherein it is indicated that high concentrations of sucrose are required to effect a protective influence. Furthermore the succinoxidase system is protected equally well by sucrose or KCl and the malic and α-ketoglutaric oxidases, both more sensitive to preparation in water than is succinoxidase (table I), are preserved in equal measure by hypertonic solutions of sucrose, glucose or mannitol (table II).

Injury done to isolated mitochondria in a hypotonic environment was more profound than that experienced by tissue pieces, and was not completely obviated even at low temperatures. The decided thermal sensitivity of the inactivation process together with the rigorous requirement for hypertonicity at the time of homogenization suggested a possible interaction between the particles and other cytoplasmic components. Such an interaction is demonstrated in figure 2 and table III.

TABLE II

THE EFFECT OF THE COMPOSITION OF HOMOGENIZING MEDIUM ON MITOCHONDRIAL ACTIVITY.

Expt.	Substrate	Preparative medium (first suspension)	Second suspension	$Q_{O_2}(N)$
1	Succinate	H_2O		396
		KCl 2.5×10^{-1} M		537
		Sucrose	Sucrose	600
		Sucrose + KH_2PO_4 + Na_2HPO_4 (pH 7.3)		520
		Mannitol		522
2	α-Keto-glutarate	H_2O	Sucrose	90
		Sucrose	Sucrose	228
		Glucose	Glucose	234
		Mannitol	Mannitol	218
3	Malate	Sucrose		485
		Sucrose + KCl + NaCl		490
		Sucrose + KH_2PO_4 + Na_2HPO_4 (pH 6.7)	As first suspension	495
		Sucrose + KH_2PO_4 + Na_2HPO_4 (pH 7.3)		452

Final concentration of reagents as in table I, high tonicity. Sucrose, glucose, mannitol all 0.5 M. Phosphate buffer in preparative medium 0.05 M, final concentration. Salt concentration in salt-sucrose mixture, 0.075 M (1 pt. KCl : 1 pt. NaCl). Sucrose concentration lowered in buffer-sucrose or salt-sucrose mixtures to give final osmolarity of 0.5 M. Final enzyme suspension in 0.5 M sucrose.

Figure 2 indicates that exposure of the particles to distilled water for a limited time, whether at 0° or at room temperature, is in itself insufficient to cause inactivation of succinoxidase. In the presence of supernatant, however, inactivation is drastic and is much greater at the higher temperature. The irreversible action of the supernatant has been shown to be non-specific, affecting both succinic dehydrogenase and cytochrome oxidase, when measured separately, as well as malic and α-ketoglutaric oxidases. Both boiling and dialysis lessen the inhibitory action of the supernatant (table III). Although the action of the supernatant may be attributed to a combination of enzymatic and non-enzymatic causes, the possibility remains that the observed effects are brought about by a heat-labile, dialyzable substance. In any event, the agent causing inactivation is not the same as the competitive inhibitor of succinic dehydrogenase found earlier in cauliflower juice (**22**). Inhibition by the latter substance is reversible and may be overcome simply by removing the bud pieces or mitochondria from the juice.

Although succinoxidase activity is not impaired by exposure of the mitochondria to water (see below for the effect upon the concomitant phosphorylation) such treatment creates a pronounced requirement for cytochrome C (table IV) which is not due to the loss of cytochrome C from the particles, but rather to a change in its physical state (**31, 35**). Despite the resistance of succinoxidase to hypotonic washing the activity of this enzyme

complex is nonetheless dependent upon the tonicity of the environment (table I, fig. 1). When additional sucrose is withheld from the manometer flask there is little apparent difference in the succinoxidase activity of particles prepared initially in water compared to that of particles prepared initially in hypertonic sucrose (table I). It appears as if one or more components of the succinoxidase system which are sensitive to destruction by the supernatant are the same components which respond to an increase in the tonicity of the medium during the respiratory measurement.

To demonstrate the effect of the tonicity of the environment upon the oxidation of succinate, mitochondrial suspensions in 0.5 M sucrose need only

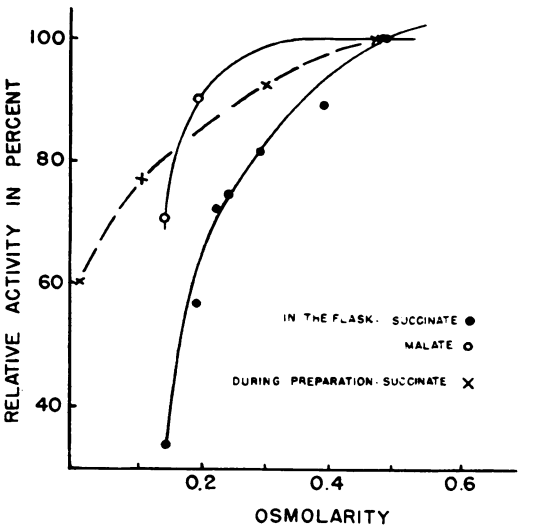

FIG. 1. The effect of the tonicity of the environment during homogenization and during the experimental period on the oxidative activity of cauliflower bud mitochondria. Final concentration of reagents as in table I, with the exception that concentration of sucrose is varied. The term *osmolarity* indicates the concentration of osmotically active components and describes the total concentration of molecular and ionic species, expressed in moles per liter. Where tonicity was varied in the flask, homogenization was carried out in 0.5 M sucrose, and where tonicity was varied during the preparation, the final osmolarity in the flask was in each case 0.5 M.

be transferred to manometer flasks containing varying quantities of sucrose. With malate as substrate, however, no stimulation of oxidation was observed upon addition of sucrose to the flask unless the enzyme aliquot contained something less than 250 μmoles sucrose (0.5 ml. of 0.5 M sucrose). Thus when 0.5 ml. enzyme suspended in 0.15 M sucrose (75 μmoles) is introduced into the manometer flask, the addition of as little as 175 μmoles sucrose will bring about near maximal rates of malate oxidation (table V, fig. 1). There appears to be no apparent injury to the malic oxidase system following passage of the particles through a small volume of 0.15 M solution.

Table I describes the effect upon succinoxidase, malic oxidase, and α-ketoglutaric oxidase, of preparation of the mitochondria in water, of pas-

TABLE III

THE EFFECT OF HEAT-TREATED OR DIALYZED SUPERNATANT ON MITOCHONDRIAL SUCCINOXIDASE.

Incubation medium	$Q_{O_2}(N)$	Per cent. control
H_2O	710	100
KCl 2×10^{-2} M	590	83
Supernatant	322	45
Boiled supernatant	500	70
Dialyzed supernatant	520	73

Final concentration of reagents as in table I, high tonicity. Substrate, succinate. Particles prepared in 0.5 M sucrose. Separately prepared water homogenate dialyzed at 2°C overnight or heated for 5 min. at 90° to 100°C. Dialyzed, boiled, and untreated water homogenate all cleared before use by centrifugation at 50,000 × g. for 45 min. in refrigerated Spinco. Incubation period 30 min. at room temperature. Particles then sedimented at 0°C and resuspended in 0.5 M sucrose.

sage of the mitochondria through a hypotonic phase, and of the tonicity of the environment during the respiratory measurement. Preparation in water is in each case deleterious, whereas passage through a hypotonic phase is apparently without consequence with respect to succinoxidase activity, but of considerable consequence with respect to both malic and α-ketoglutaric oxidase activity. The tonicity in the flask is in each case an important determinant of the final oxidative rate, and the tonic requirements vary with the different oxidases.

It will be shown below that addition of dilute salt to hypotonic bathing media may preclude injury to the malic and α-ketoglutaric oxidases, when such injury would be manifest following suspension of the mitochondria in distilled water. However, preliminary experiments established the fact that dilute salt does not obviate the damage done by initial homogenization in distilled water. Furthermore, the addition of salt or of buffer is without effect when supplied in addition to sucrose during the initial grinding (table II), in distinction to the observations of MILLERD (**25**) with mitochondrial preparations from mung beans. The susceptibility of malic and α-ketoglutaric oxidase to hypotonic treatment is not related to the requirement of these systems for exogenous adenylates or pyridine nucleotides (**21**), for these oxidases have been shown to respond to an increase in tonicity whether or not additional cofactors were provided.

OSMOTIC INJURY IN THE ABSENCE OF SOLUBLE CELL COMPONENTS

Since one or more soluble protoplast components were shown to bring about the inactivation of several mitochondrial oxidases under hypotonic conditions, those cases were reexamined which showed impairment of oxidase activity following exposure to distilled water (table I), to determine whether residual supernatant was responsible for the observed inactivation. To test this possibility, particles were twice centrifuged in 0.5 M sucrose prior to a third centrifugation in water. Particles so prepared were compared to mitochondria undergoing hypotonic washing during the second

centrifugation, as usual, followed by a third suspension and centrifugation in 0.5 M sucrose. Surprisingly the inactivation caused by the first sequence above was always considerably greater than that caused by the second (table VI, Experiment 1). Residual supernatant was therefore ruled out as the cause of inactivation.

Further experiments indicated that the relative advantage of the second sequence was not to be explained in terms of recovery of the particles in hypertonic solution following exposure to water. If the first washing series was modified so that the third suspension was carried out in a small volume of water (2.0 ml.) instead of in the usual large volume (30 ml.), the inacti-

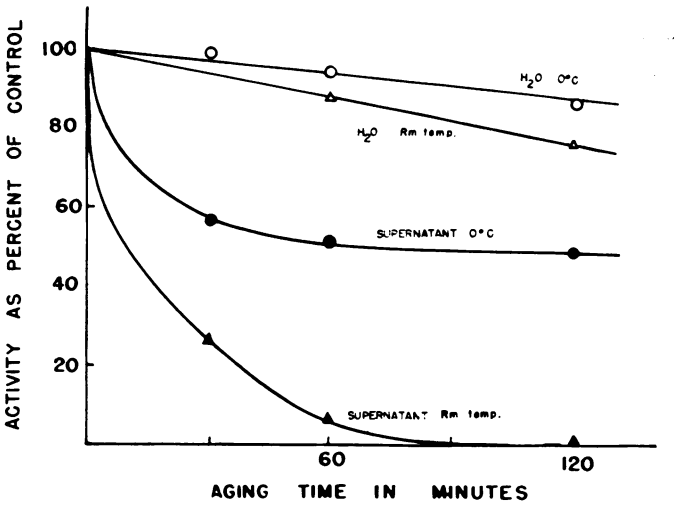

FIG. 2. The relative effect of distilled water and protoplast components on cauliflower succinoxidase. Final concentration of reagents as in table I. Particles aged in water were prepared in 0.5 M sucrose. Where treatment with supernatant is indicated, particles were prepared from aged water homogenates. Controls prepared in sucrose, and activity measured at once.

vation normally caused by water washing was sharply reduced (table VI, Expt. 2).

It appeared unlikely that the small quantity of sucrose to be found in the precipitate following the second centrifugation afforded osmotic protection when this precipitate was resuspended in a small volume of water, since the washing of particles in a large volume of 0.15 M sucrose was almost as detrimental to malic oxidase as washing in a large volume of water. An explanation was therefore sought in terms of the dilution during large volume washing of some factor necessary to the integrity of the mitochondrial enzymes in question.

When a small-volume water suspension of mitochondria was centrifuged and the supernatant removed, the particles were subsequently active in the oxidation of malate, whereas if a similar suspension was dialyzed they were not. Thus injury was attributed to hypotonicity in the absence of certain

TABLE IV

THE CYTOCHROME C REQUIREMENT OF MITOCHONDRIAL SUCCINOXIDASE.

Homogenizing medium	Resuspending medium	$Q_{O_2}(N)$		Per cent. increase due to cytochrome
		− Cytochrome c	+ Cytochrome c	
H_2O	Sucrose	84	250	198
Sucrose	H_2O	109	398	265
Sucrose	Sucrose	313	398	27

Final concentration of reagents as in table I, high tonicity. Succinate as substrate. Final suspension of particles in 0.5 M sucrose in all cases.

soluble components normally present in a mitochondrial suspension. Neutral salts were first tested for possible identity with the soluble factor in question, simply because it was reasonable to envisage the presence of such salts in bud homogenates, and to consider that such salts would be readily susceptible to being washed away. When particles were suspended in as low as 10^{-3} M KCl instead of in water during the third centrifugation (following two centrifugations in 0.5 M sucrose), inactivation of both malic and α-ketoglutaric oxidase was largely precluded (tables VI and VII). This sparing action appears to be unspecific insofar as the potassium or sodium salts of chloride or phosphate are equally effective (table VI). In but one or two instances the suspension of particles in neutral salt in small volume has proved deleterious, and these aberrations remain unexplained.

In the experiments described above, hypotonicity *per se* was not sufficient to bring about the observed inactivation in relatively short incubation periods. For damage to occur, hypotonicity must be imposed in the absence of low levels of inorganic salt. This explains why hypotonic treatment following the first centrifugation in sucrose is not as devastating as similar treatment following two hypertonic washings. In the latter instance the salt level has been more rigorously depleted before the subsequent exposure to

TABLE V

THE RELATION OF MALATE OXIDATION TO AMBIENT TONICITY.

Experiment	Final suspension[1] (2.0 ml.)	Sucrose addendum to flask (millimoles)		
		0.0	0.175	0.9
1	0.5 M sucrose	485	....	483
	H_2O	258	....	385
	0.01 M KCl	345	....	459
2	0.5 M sucrose	350	....	372
	0.15 M sucrose	283	338	376
3	0.15 M sucrose	283	394	417

[1]First and second suspensions in 40 and 30 ml. 0.5 M sucrose respectively.

Reagent concentrations as in table I, high tonicity, with l-malate as substrate. In addition, glucose, 10^{-2} M final concentration [as substrate for endogenous hexokinase (21)].

TABLE VI

THE EFFECT OF DILUTE SALT ON THE OSMOTIC INACTIVATION OF MALIC OXIDASE.

Experiment	Second suspension	Third suspension[1]	$Q_{O_2}(N)$
1	Sucrose	Sucrose	570
	H_2O	Sucrose	392
	Sucrose	H_2O	119
2	Sucrose	Sucrose	316
	Sucrose	H_2O	46
	Sucrose	Sucrose (2.0 ml.)	458
	Sucrose	H_2O (2.0 ml.)	324
3	Sucrose	Sucrose	458
		H_2O	218
		KCl[2]	430
		NaCl	464
		K phosphate, pH 7.0	434
		Na phosphate, pH 7.0	487
4	Sucrose	H_2O	123
		4×10^{-4} M KCl	181
		2×10^{-3} M	294
		1×10^{-2} M	362
		5×10^{-2} M	360

[1]Volumes of all suspensions 30 ml, except where specified.
[2]Salts 2×10^{-3} M.
Reagent concentration as in table I, high tonicity, with l-malate as substrate. In addition, glucose, 10^{-2} M final concentration. First and final suspension in 0.5 M sucrose in all cases. Final suspension 2.0 ml.

distilled water. Hypotonicity is clearly a prerequisite to damage of the particles. Osmotic injury may conceivably follow the exposure of normally protected areas of the mitochondrion to the environment, or may be the consequence of leaching of ions which in hypertonic solution would not be washed away. Whatever the process, it appears irreversible. If the mitochondria are distended in water and dilute salt is then added before the suspension is made hypertonic again, damage is not diminished.

THE RELATIVE EFFECTS OF HYPOTONICITY ON OXIDATION AND PHOSPHORYLATION

Numerous reports (**23, 32, 33**) have indicated that the oxidative and phosphorylative capacities of mitochondria are susceptible in different degree to inactivation by hypotonic treatment. In view of the above findings that hypotonicity *per se* frequently failed to bring about the impairment of oxidative activity, it was deemed interesting to investigate the effect of hypotonicity on both oxidation and phosphorylation under conditions where hypotonicity was achieved both in the presence and absence of neutral salts.

Following two washings in 0.5 M sucrose in the centrifuge, particles were suspended in a large volume (25–30 ml.) of water or 5×10^{-3} M KCl, and

centrifuged again. The oxidative and phosphorylative capacities of particles so treated were compared to the corresponding activities of particles washed for the third time in sucrose-KCl (table VII). It is interesting that whereas the oxidation of succinate is affected but to a limited extent by submitting the particles to a large volume of distilled water, the concomitant

TABLE VII

THE EFFECT OF HYPOTONICITY ON OXIDATION AND PHOSPHORYLATION AS RELATED TO THE SALT CONTENT AND VOLUME OF THE SUSPENDING MEDIUM.

Expt.	Substrate	Third suspension[1] (25 ml.)	Final suspension (2.0 ml.)	$Q_{O_2}(N)$	P/O
1	α-Ketoglutarate	Sucrose-KCl		179	1.48
		H_2O	Sucrose	42	1.11
		KCl		159	1.19
2	α-Ketoglutarate	Sucrose-KCl		174	1.43
		H_2O	Sucrose	57	0.81
		KCl		167	1.63
	Succinate	Sucrose-KCl		234	1.42
		H_2O	Sucrose	197	0.0
		KCl		228	0.42
3	Malate	....	H_2O, incubated	337	1.55
		....	Sucrose, incubated	348	1.59
		H_2O	Sucrose	171	1.05
		KCl	Sucrose	281	1.07
4	Succinate	....	H_2O, incubated	183	0.0
		....	Sucrose, incubated	279	1.34
		H_2O	Sucrose	117	0.0
		KCl	Sucrose	119	0.62
5	α-Ketoglutarate	....	H_2O, incubated	179	2.36
		....	Sucrose, incubated	220	2.16
		H_2O	Sucrose	59	0.77
		KCl	Sucrose	162	2.03

[1]Sucrose 0.5 M, KCl 10^{-3} M.

Reagent concentrations as in table I, high tonicity. In addition, glucose, 10^{-2} M final concentration; NaF, 10^{-2} M; and yeast hexokinase (sufficient to phosphorylate 0.5 μmole glucose minute). Sufficient 1.5 M sucrose added to water suspensions following pre-experimental incubation to make final concentration 0.5 M. Incubation 20 min. at 0°C where indicated. P/O ratio indicates μmoles inorganic phosphate esterified per μatom oxygen utilized.

phosphorylation is completely abolished. The presence of neutral salt exerts some small protection of the largely refractory oxidative system and very little protection of the phosphorylative system. With α-ketoglutarate as substrate, however, the inactivation of the oxidative system caused by passage of the particles through a large volume of water is considerable, and the sparing action of neutral salt is also large. Although phosphorylation

is diminished to some extent by water treatment, the diminution is but slightly in excess of that exhibited by the respiration, and there is evidence of sparing or protection of the phosphorylative machinery by neutral salt.

Hypotonicity in small volume was shown to have but a limited influence upon respiration (table VI). In experiments 3 to 5 of table VII the effect upon oxidation and phosphorylation of exposure of particles to small volumes of water is compared directly to the effects of large volume washing in water or dilute salt. Neutral salt protects both the oxidative and phosphorylative systems when α-ketoglutarate is given as substrate, whereas with malate a greater sparing action is exerted upon the oxidative system. The phosphorylative mechanisms associated with succinate oxidation appear to be particularly sensitive to hypotonicity, being made completely inoperative by both small and large volumes of distilled water, and being spared but little by neutral salt. Small-volume exposure to distilled water appears to exert but a limited effect upon the oxidative and phosphorylative characteristics of malic and α-ketoglutaric oxidase.

Discussion

In much of the work relating mitochondrial structure to function (**12, 13, 14**) the inactivation of certain mitochondrial enzyme systems brought about by hypotonic environments has been attributed neither to leaching nor to dilution effects, but rather to the alteration of requisite spatial configurations. CLAUDE (**5**) has suggested the existence of a mitochondrial membrane which may be modified or even disrupted in a hypotonic environment. Although the existence of such a membrane has recently been reaffirmed by excellent electron microscope studies (**30**) much evidence militates against the view that effects upon the membrane are causal to the gross enzymatic changes observed.

The experiments presented above have suggested that under certain conditions the swelling of the mitochondria in hypotonic solution may not in itself be responsible for injury to the oxidative and phosphorylative systems, but may be a prerequisite to such injury. At least two distinctly different destructive effects have been described for which hypotonicity is necessary but not sufficient. A type of injury at least in part non-enzymatic is indicated which is attributable to the action of one or more soluble components of the protoplast upon several of the mitochondrial enzymes. Succinic dehydrogenase, cytochrome oxidase, malic and α-ketoglutaric oxidases, and succinoxidase are all susceptible to this type of inactivation. The effect upon malic, α-ketoglutaric, and succinic oxidase is in each case greater than can be accounted for by the effect upon the cytochrome oxidase system. This type of inactivation is largely precluded by carrying out the initial homogenization of tissue in 0.5 M sucrose, in the cold. The instantaneous injury suffered by the mitochondria when cauliflower buds are homogenized in water indicates the osmotic response of mitochondria to be even more rapid than previously suggested (**29**) and clearly distinguishes this type of

injury from the irreversible changes effected by hypotonic exposure in excess of fifteen minutes (**12**).

In an early study of the effect of tonicity upon certain oxidative systems, POTTER (**31**) described a situation wherein rat liver cells washed isotonically and then exposed to distilled water were able to oxidize octanoate, whereas cells first exposed to distilled water and then washed were not. This anomaly was interpreted in terms of the removal of an unknown necessary factor by washing following lysis. However it may well be that the situation is comparable to that found here with cauliflower preparations.

When cauliflower mitochondria are once removed from the supernatant solution following centrifugation, the succinoxidase activity is shown to be little affected by exposure to distilled water. The malic and α-ketoglutaric oxidase activity also survives such exposure when suspension of the mitochondria is done in small volume. When exposure to distilled water is carried out in larger volume, succinate oxidation is still not greatly affected, whereas the oxidation of malate and α-ketoglutarate is appreciably diminished. However, if neutral salt in concentration as low as 1 to 5×10^{-3} M is substituted for water, this inactivation is largely precluded.

Although hypotonicity has been considered a sufficient condition to evoke enzymatic changes within mitochondria, CLAUDE (**5**), in studying the effect of the ambient tonicity on the structure and chemical constitution of the large granules (primarily mitochondria) of guinea pig liver, has made the distinction between hypotonicity *per se* and hypotonicity in the absence of salts. The latter condition was observed to bring about the immediate disintegration of the large granules which otherwise were seen to change their structure more slowly. The comparative impunity with which small-volume washing of cauliflower mitochondria may be effected, observed here with respect to enzymological properties, is comparable to the structural immunity of the large granules observed by Claude under similar conditions. That qualitatively distinct types of structural changes may occur, and that these changes may variously affect the enzymatic characteristics of the mitochondria, has been emphasized by HARMAN (**12**), although no consideration was given in the latter work to the absence of salt in an environment of low osmotic pressure as a condition distinct from hypotonicity *per se*. It will be of interest to learn whether the salt requirement is for the integrity of the particles only, or whether the requirement is exhibited as well by the solubilized systems (**9**).

The phosphorylative and oxidative characteristics of cauliflower bud particles are differently affected by hypotonicity. The phosphorylations accompanying succinate oxidation are completely obviated by hypotonic treatment both in small and in large volume, and little protection of this phosphorylative activity is afforded by neutral salt. The phosphorylation related to α-ketoglutarate oxidation is destroyed only by large volumes of distilled water, and this destruction is precluded by neutral salt. Phosphorylation accompanying malate oxidation, in turn, appears to be in an

intermediate category, being sensitive to exposure to large volumes of distilled water, yet incompletely protected by neutral salt.

Although particles may obviously begin to swell within five minutes when placed in hypotonic medium (**29**), irreversible injury to the cyclophorase system of rabbit liver has been considered to be a consequence of hypotonic exposure in excess of fifteen minutes (**12**). It is significant that in the presence of salt the oxidative and phosphorylative systems concerned with malic and α-ketoglutaric acid metabolism are unaffected by exposures in excess of 15 minutes, although the effect upon the cyclophorase system in its entirety has not been studied.

It is not suggested that in the experiments above the extent to which a process is disrupted by exposure of the mitochondria to hypotonic medium has any quantitative significance. That is, the extent of the inhibition of respiration or phosphorylation may well have been either greater or less depending upon variations in temperature, duration of exposure and perhaps upon the biological material. However, the qualitatively different behavior of the succinoxidase system compared to the α-ketoglutaric and malic oxidase systems seems significant. That is, the interesting situation obtains wherein the succinoxidase system is almost insensitive to exposure to a hypotonic environment, whereas the associated phosphorylative system is completely destroyed by such a procedure. On the other hand, malic and α-ketoglutaric oxidase are both sensitive to a hypotonic environment and the associated phosphorylative systems are susceptible to approximately the same extent as the oxidative processes. It would appear that the succinoxidase activity does not depend upon concomitant phosphorylation, whereas malic and α-ketoglutaric oxidase activity does.

More than one type of phosphorylative mechanism is indicated. Furthermore it would seem that more than one of the phosphorylations accompanying malate or α-ketoglutarate oxidation occur at loci not shared with the succinoxidase system, since the P/O ratios observed during the oxidation of either malate or α-ketoglutarate, by particles previously suspended in small volumes of distilled water, are well above 1.0, whereas the same ratio for succinate oxidation under similar circumstances is zero. Some indication of the existence of more than one type of phosphorylative mechanism has already been given by HUNTER (**16**) who showed that the phosphorylation accompanying the primary oxidation of α-ketoglutarate is insensitive to 2,4-dinitrophenol (DNP), whereas the phosphorylations accompanying electron transfer from reduced coenzyme I onward are DNP sensitive.

In all probability the P/O ratios obtained in the foregoing studies are lower than could be expected. The experimental values suggest a reasonable probability of 2.0 for succinic oxidation and 3.0 for malic and α-ketoglutaric acid oxidation. The P/O ratio for α-ketoglutaric acid oxidation may be even greater than 3.0 (**16**). Thus, in the studies described above, some part of the phosphorylative system is already inoperative at the time of the measurement as a result of the preparative technique, and it is impossible

to accurately describe quantitatively the effect of the imposed environmental treatment on the various phosphorylative systems.

BONNER and MILLERD (**3**) have carried out extensive studies pertaining to the ability of mitochondria prepared from mung beans to effect oxidative phosphorylation. The P/O ratios obtained in the latter studies have been predominantly close to 1.0, although occasional experiments have yielded ratios close to 3.0 for the oxidation of α-ketoglutarate. In view of the variable lability of the phosphorylative systems described in the experiments presented here, and the occasional high ratios observed by the above authors, it seems likely that ratios in mung beans will ultimately prove to be above 1.0. Mung beans perhaps represent a material in which uncoupling is more readily effected during enzyme preparation than is the case with cauliflower buds.

When particles have suffered no irreversible injury during the preparation or washing, the activity of the mitochondrial enzymes is dependent upon the tonicity in the manometer flasks. Similar observations have been made by others (**7, 24**). The tonicity requirements appear different for the oxidation of different substrates and, interestingly, succinoxidase, the oxidative system least sensitive to hypotonic treatment during the preparation, responds most perceptibly to an increase of tonicity in the flask. In spite of the fact that the effect of the ambient tonicity upon mitochondrial function is frequently indirect, there is compelling evidence that the tonicity *per se* is of great consequence, both with respect to maintaining certain phosphorylative systems intact, and with respect to the achievement of maximal oxidative activity.

Summary

The effect upon cauliflower bud mitochondria of preparation in water, of passage through a hypotonic phase, and of the tonicity of the environment during the experimental period has been investigated with respect to the oxidative and phosphorylative capacities of the succinoxidase, malic oxidase, and α-ketoglutaric oxidase systems.

Two types of enzymatic inactivation have been described for which hypotonicity is a necessary but insufficient condition. The exposure of cauliflower bud mitochondria to a hypotonic environment in the presence of soluble components of the protoplast results in the rapid and irreversible deterioration of the malic, α-ketoglutaric and succinoxidase systems. Such destruction may be prevented by the preparation of mitochondria in cold hypertonic solution.

When mitochondria are suspended in distilled water, in volumes commonly employed, in the absence of other protoplast components, the succinoxidase system remains unaffected, whereas the malic and α-ketoglutaric oxidase systems suffer considerable injury. This type of injury is minimized or precluded either by the substitution of dilute neutral salt solutions for distilled water, or by suspension of the mitochondria in small volumes of distilled water.

The phosphorylative system in association with the succinoxidase complex is completely destroyed by any exposure to a hypotonic environment, whether or not neutral salts are present. On the other hand the phosphorylative machinery in association with malic and α-ketoglutaric oxidase is affected to approximately the same extent as the oxidative systems by hypotonic exposure, and both the oxidative and phosphorylative systems are protected by neutral salt. The different effect of low tonicity upon the P/O ratios observed with various substrates has led to the suggestion that more than one of the phosphorylations accompanying malate or α-ketoglutarate oxidation occur at loci not shared in common with the succinoxidase system.

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