10. RAMSEY, J. B. and COLICHMAN, E. L. The potentiometric determination of vitamin C. Ind. Eng. Chem., Anal. Ed. 14: 319–321. 1942.
11. ROBERTSON, T. S. B. The accumulation of vitamin C during germination of peas. Australian Jour. Exptl. Biol. Med. Sci. 25: 41–46. 1947.
12. RONDONI, P. and CUDKOWICZ, G. Hydrogen peroxide in tumors. Experientia 9: 348–353. 1953.
13. SPIKES, JOHN D. Stoichemistry of the photolysis of water by illuminated chloroplast fragments. Arch. Biochem. Biophys. 35: 101–109. 1952.
14. TALARICIO, G. Comportamento della catalisi del fegato alle luci monochromatische. Arch. Pharmacol. Sper. 8: 81–109. 1909.
15. VOSKRESENSKAYA, N. P. The effect of conditions of illumination on the composition of the products of photosynthesis. Trudy Inst. Fiziol. Rastenii, im. K. A. Timiryazeva, Akad. Nauk S.S.S.R. 8: 1 (1953): 42–56. 1953.

EFFECTIVENESS OF ETHYLENE DIAMINE TETRA-ACETIC ACID IN THE ACTIVATION OF OXIDATIONS MEDIATED BY MITOCHONDRIA FROM BROCCOLI BUDS[1]

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Mitochondria prepared from broccoli buds by the usual procedure, using 0.5 M sucrose as the homogenizing medium, showed poor activity. The rate of oxidation of succinate rapidly declined with time and there was virtually no oxidation of α-ketoglutarate. Several workers, with both plant (10, 16) and animal (3, 14) material, reported that disodium EDTA (disodium ethylene-diamine tetra-acetic acid) added to the preparative medium activated and stabilized mitochondrial oxidations. This type of activation was obtained with mitochondria from broccoli buds. The object of the present study was to determine the effectiveness of EDTA on broccoli mitochondria and to obtain clues as to the mechanism of activation.

MATERIALS AND METHODS

With some modifications the method outlined by Laties (9) was used to prepare the mitochondria. In the preparative procedures all manipulations were carried out at 0° C. The broccoli buds were cut off the flower stalks and 12 gm were ground in a mortar with 20 gm of fine acid-washed sand and 25 ml of either 0.5 M sucrose or 0.5 M sucrose containing 0.01 M EDTA. Additional 10-ml portions of preparative medium were added to aid in homogenizing the tissue. Finally the homogenate was diluted with the same medium to make a total volume of 90 ml containing the 12 gm of tissue.

The resulting homogenate was squeezed through four layers of cheesecloth and the filtrate centrifuged at $1000 \times g$ for 5 minutes to precipitate pollen grains, unbroken cells, nuclei, cell debris and sand. The supernatant was then centrifuged at $17{,}000 \times g$ for 15 minutes. The second precipitate, which yielded the mitochondrial pellet, is described as "once washed".

A "twice washed" preparation was one in which the pellet was resuspended in 20 ml of 0.5 M sucrose and homogenized in the centrifuge tube with a snug-fitting lucite pestle. The resuspended particles were spun down at $17{,}000 \times g$ for 15 minutes. This procedure was repeated once more to give a "thrice washed" preparation. After all washes the final mitochondrial pellet was suspended in 4 ml of 0.5 M sucrose and homogenized with a motor-driven Teflon pestle.

The oxidative capacity of the mitochondria was determined by their ability to oxidize α-ketoglutarate or succinate. This was assayed at 25° C by standard Warburg manometric techniques. Details as to the content of the reaction mixtures are given in the legend for each figure.

Each experiment was replicated at least 3 or 4 times and the data are averages of these experiments. The data are reported in microliters oxygen per milligram nitrogen per hour designated as $Q_{O_2}(N)$.

RESULTS

ACTIVATION AND STABILIZATION EFFECT OF EDTA: The effect of isolating mitochondria in 0.5 M sucrose containing 0.01 M disodium EDTA and in 0.5 M sucrose is shown in figures 1 and 2. With α-ketoglutarate as substrate the preparation without EDTA showed very low activity ($Q_{O_2}(N)$ of approximately 40). The preparation with EDTA exhibited a four-fold increase in oxidation ($Q_{O_2}(N)$ of approximately 160), which was maintained for at least 2 hours. A similar activation was shown with succinate as a substrate (fig 2). The most striking effect, especially with succinate, was the stabilization of activity for at least a 2-hour period.

EFFECT OF WASHING ON THE ACTIVATION EFFECT OF EDTA: The purpose of the washing experiments was to reveal whether the activation affected by preparation in EDTA could be reversed by removal of the EDTA. In these experiments the activity of EDTA-prepared mitochondria washed once was compared with preparations washed twice and thrice with 0.5 M sucrose. Removal of EDTA by washing did not

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reverse the activation effect (fig 3). The EDTA in the mitochondria and the supernatant before and after washing was determined by the method of Darbey (5). In an aliquot calculated to have 3.7 mg of EDTA the first washing contained 3.5 mg and no EDTA was retained in the mitochondria. These data indicated that all the EDTA was washed out of the mitochondria in the first washing.

From these data it appears that once the mitochondria are prepared in EDTA they cannot be inactivated by removal of the EDTA. It is also evident that the EDTA is completely removed by washing. The activation can, therefore, be interpreted as due to a chelation effect which does not require the presence of EDTA after sequestering has occurred.

Comparison of an Active and Inactive Form of EDTA: In order to determine whether the EDTA molecule as such is effective regardless of the nature of the attached cation, the action of ferric EDTA was compared with that of disodium EDTA. Disodium EDTA has a complex formation constant (K) of approximately 1.6, while ferric EDTA has a K of 25.1 (11, 12). Consequently ferric EDTA is very feebly dissociated and cannot be displaced by any of the common cations (11, 12). For all practical purposes ferric EDTA could not be considered a chelating agent of any of the common cations found in a cell. It was therefore interesting to determine its effect on the mitochondria as compared with that of disodium EDTA.

The results of these experiments are given in figure 4. These data clearly show that ferric EDTA does not activate the mitochondria, while disodium EDTA markedly activates them. Since both sub-

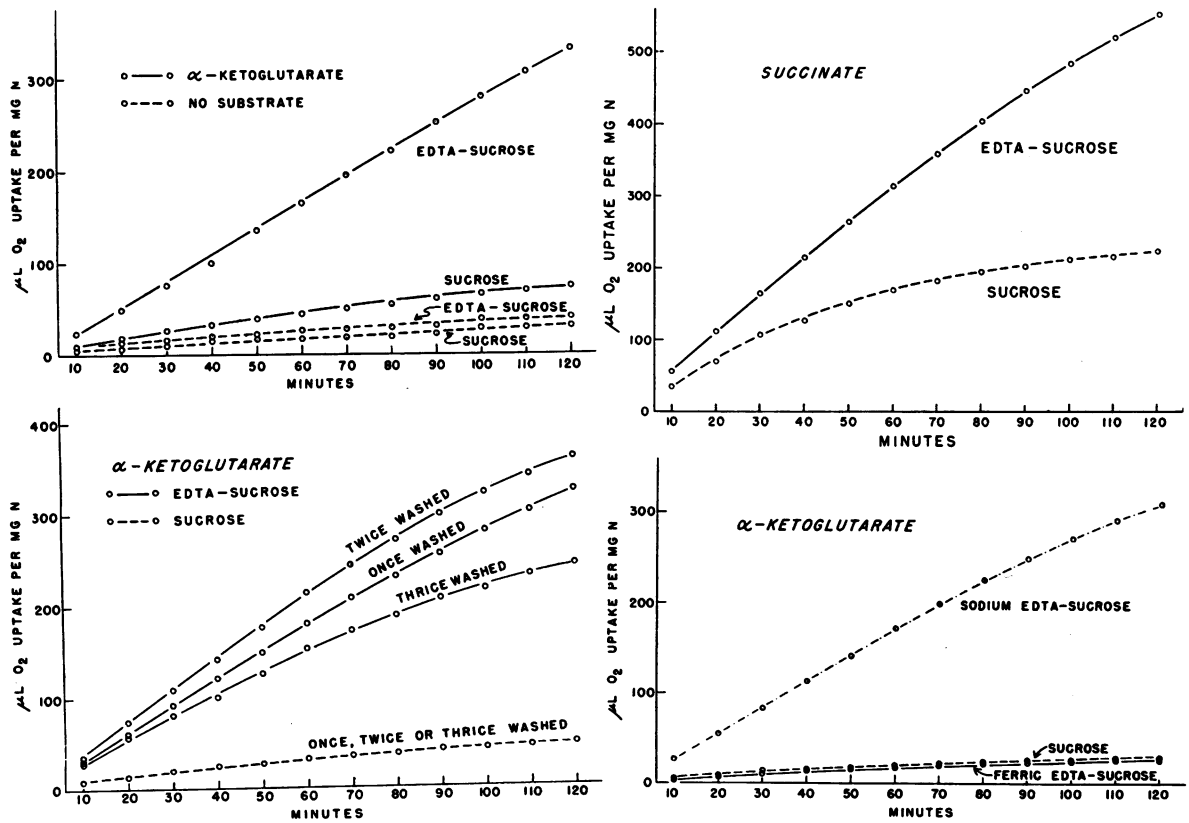

Fig. 1 (*upper left*). Oxidation of α-ketoglutarate by mitochondria from broccoli buds isolated in 0.5 M sucrose medium and in a medium containing 0.01 M EDTA in 0.5 M sucrose at 25° C. Reaction mixture contained 0.001 M ATP, 0.006 M Mg^{++}, 0.02 M glucose, 0.5 M sucrose, 0.02 M α-ketoglutarate, and 0.5 ml mitochondrial suspension containing approximately 1.0 mg nitrogen.

Fig. 2 (*upper right*). Oxidation of succinate by mitochondria from broccoli buds isolated in a 0.5 M sucrose medium and in a medium containing 0.01 M EDTA in 0.5 M sucrose at 25° C. Reaction mixture additions were the same as for figure 1 except for substrate.

Fig. 3 (*lower left*). Comparison of oxidative activity of once-, twice-, and thrice-washed preparations of mitochondria isolated in a 0.5 M sucrose medium and in a medium containing 0.01 M EDTA in 0.5 M sucrose at 25° C. Reaction mixture contained same additions as in figure 1.

Fig. 4 (*lower right*). Comparison of oxidative activity of mitochondria prepared in 0.01 M disodium EDTA in 0.5 M sucrose, 0.01 M ferric EDTA in 0.5 M sucrose, and in 0.5 M sucrose, at 25° C. Reaction mixture contained same additions as in figure 1.

TABLE I

EFFECT OF ADDING DISODIUM EDTA TO REACTION MIXTURE* ON OXIDATIVE ACTIVITY

PREP MEDIUM	NO. OF WASHES	ADDITION OF 0.001 M EDTA TO REACTION MIXTURE	$Q_{O_2}(N)$
0.5 M Sucrose	1	–	36
" "	1	+	37
0.5 M Sucrose	2	–	34
" "	2	+	41
0.5 M Sucrose	3	–	29
" "	3	+	23
0.5 M Sucrose-0.1 M EDTA	2	–	213
" " " "	2	+	232

* Reaction mixture contained same additions as in fig. 1.

stances are salts of EDTA which differ mainly in their capacity to chelate, the activation effect apears to be due to chelation.

EFFECT OF EDTA AFTER ISOLATION OF MITOCHONDRIA IN 0.5 M SUCROSE: Experiments were performed to determine the effectiveness of disodium EDTA in the reaction mixture containing mitochondria prepared with and without EDTA.

It appears from the data shown in table I that 0.001 M disodium EDTA added to the reaction mixture, after isolating the mitochondria in 0.5 M sucrose, has no beneficial effect on oxidation. Addition of 0.001 M disodium EDTA to the reaction mixture after isolating the mitochondria in EDTA had no additional effect. Apparently the chelation which enhances oxidation by the mitochondria must take place during the time the cells are broken. Otherwise the mitochondria are irreversibly inhibited.

DISCUSSION

As a result of this study the generalization can be made that active mitochondria with a stabilized rate of activity could be obtained only by isolating in an EDTA-sucrose medium. It must be pointed out, however, that seasonal variability of the tissue was considerable and during the summer and early fall the stimulatory effect of EDTA was not obtained. In these cases the activity of the isolated mitochondria was extremely low, probably because of factors unrelated to the action of EDTA. The data reported here were obtained from material secured during the winter and spring months.

The simplest explanation of the data herein reported is to assume that EDTA stimulates activity by chelation of inhibitory cations. It is not known specifically what cation or cations are chelated. One can, however, postulate that the deleterious cations, released into the homogenate when the cells are broken, are being sequestered. An example of this type of inhibition is the oxidation of SH groups by heavy metals. In the living cell these cations may be combined in linkages which make their presence innocuous. The physiological shock of cell rupture or the mixing of vacuolar sap with cytoplasmic material may cause hydrolysis of the linkage which binds the cation to its endogenous partner. When freed in the homogenate these cations may become attached to enzymes or cofactors in a manner which inhibits them. However, when the cells are broken in the presence of disodium EDTA, the deleterious ions are chelated and the mitochondria are spared. Once homogenization in the absence of EDTA has occurred, the mitochondria are irreversibly inactivated.

The reports on the use of EDTA with plant tissues are variable. Price and Thimann (10) observed that the addition of EDTA to the extraction medium was of little consequence with mitochondria from etiolated pea stems, but with further purification a definite beneficial effect was shown in succinoxidase activity. Biale and Young (2), using avocado tissue, and Sharpensteen and Conn (13), using white potato, found no beneficial effect of EDTA. Tager (16) reported considerable enhancement of pyruvate oxidation by mitochondria from Avena seedlings prepared in sucrose solutions containing EDTA. From these reports it appears that while EDTA is effective with some plant tissues, it is only partially effective with others and totally ineffective with still others.

Apparently the beneficial effect of EDTA is more universal with animal tissues. Slater and Cleland (14) preserved the activity of heart sarcosomes at temperatures of 15 to 25° C by addition of EDTA to the sarcosomal suspension. The best sarcosomal preparations were obtained by including disodium EDTA in the isolation medium. However, these sarcosomes were very susceptible to inactivation by added calcium ions. Since calcium is known to stimulate ATP-ase (1), Slater and Cleland's concept (15) is that calcium is adsorbed on the sarcosomes during isolation and subsequently causes hydrolysis of the ATP in the membrane. Consequently there is a loss of the high-energy phosphate, which is presumably needed to maintain the stability of the mitochondrial membrane. The presence of EDTA in the isolating medium or in the suspension serves to chelate calcium and thereby prevents the inactivation.

Gross (8) demonstrated that EDTA in concentrations of 0.001 to 0.003 M accelerates ATP-ase activity, but in concentrations above 0.003 M inhibited ATP-ase by chelation of calcium present in a concentration of 0.003 M. The acceleration at lower concentrations of EDTA was attributed to chelation of inhibiting trace metals. The work of Friess (6), Friess et al (7), and Bowen and Kerwin (4) on the effect of EDTA on ATP-ase may have considerable bearing on the concepts of Slater and Cleland (15) and Bonner (3).

Bonner (3) found that succinoxidase preparations from heart muscle, inactivated by various means, could be reactivated by addition of 10^{-4} M EDTA. This was apparently not a chelation effect since inactivation occurred when EDTA was removed from the

preparation by washing or dialyzing against trace-metal-free buffer solutions. Addition of EDTA to this inactivated preparation reactivated it. Inactivation was thus reversible, depending on the presence or absence of EDTA. These data indicate that the activation effect of EDTA might be due to a direct action on the enzyme.

It should be noted that Bonner's succinoxidase system was not a standard mitochondrial preparation. The reversible activation by EDTA in this preparation can be explained by assuming that the inhibiting cation is very firmly fixed to the protein structure and cannot be removed by dialysis. Therefore, EDTA can only be effective when it is in contact with the protein and can mask or shield the enzyme from the inhibiting cation.

Apparently in some tissues the deleterious cations are very firmly bound to the protein complex. In these cases EDTA must be present to block the inhibiting cations. Other tissues, such as broccoli, may have mobile or loosely held inhibiting cations which can be more easily sequestered. In this latter case the effect of EDTA is non-reversible as shown earlier. The avocado (2) may be an example of a third type of tissue in which EDTA has no observable effect. This tissue may be one in which the cation concentration is so low that further chelation is ineffective.

Summary

1. Preparation of broccoli mitochondria in a medium containing 0.01 M disodium EDTA in 0.5 M sucrose stimulates the oxidation of α-ketoglutarate and succinate two- to fourfold and also stabilizes the rate of oxidation during a 2-hour period.

2. a) Removal of EDTA from activated mitochondria by washing did not inactivate them. b) The use of ferric EDTA, a stable salt which has practically no chelating properties, did not cause activation. c) Addition of disodium EDTA to the reaction mixture containing sucrose-prepared mitochondria did not activate them.

3. The data presented indicate that chelation of deleterious cations is involved in the mode of action of disodium EDTA. Once contamination by the deleterious factors has occurred, the inactivation is irreversible with EDTA.

Literature Cited

1. Bailey, K. Myosin and adenosinetriphosphatase. Biochem. Jour. 36: 121. 1942.
2. Biale, J. B. and Young, R. E. Effects of EDTA on oxidation mediated by avocado mitochondria. Abstract of papers, Western Section, Amer. Soc. Plant Physiol. June meeting, 1954.
3. Bonner, W. D. Activation of the succinic dehydrogenase-cytochrome system. Biochem. Jour. 56: 274–285. 1954.
4. Bowen, W. J. and Kerwin, T. D. A study of the effects of EDTA on myosin ATP-ase. Jour. Biol. Chem. 211: 237–247. 1954.
5. Darbey, A. Colorimetric determination of sodium salts of EDTA. Anal. Chem. 24: 373–378. 1952.
6. Friess, E. T. The effects of a chelating agent on myosin ATP-ase. Arch. Biochem. Biophys. 51: 17–23. 1954.
7. Friess, E. T., Morales, M. F., and Bowen, W. J. Some further observations on the interaction of EDTA with myosin-ATP system. Arch. Biochem. Biophys. 53: 311–313. 1954.
8. Gross, M. Effect of EDTA on ATP-ase activity. Science 118: 218–219. 1953.
9. Laties, G. C. The physical environment and oxidative and phosphorylative capacities of higher plant mitochondria. Plant Physiol. 28: 557–575. 1953.
10. Price, C. A. and Thimann, K. V. The estimation of dehydrogenases in plant tissue. Plant Physiol. 29: 113–124. 1954.
11. Schwarzenbach, G. and Ackermann, H. Die Athylendiamin-tetraessigsaure. Helv. Chim. Acta 30: 1798–1804. 1947.
12. Schwarzenbach, G. and Heller, J. Die Eisen (II) und Eisen (III) Komplexe der Athylendiamin-tetraessigsaure und ihr Redoxgleichgewicht. Helv. Chim. Acta 34: 576–591. 1951.
13. Sharpensteen, H. H. and Conn, E. E. Preparation and properties of potato mitochondria. Abstract, 29th Ann. Meeting Amer. Soc. Plant Physiol. P. 37. Sept. 1954.
14. Slater, E. C. and Cleland, K. W. Stabilization of oxidative phosphorylation in heart muscle sarcosomes. Nature 170: 118–119. 1952.
15. Slater, E. C. and Cleland, K. W. The effect of calcium on the respiratory and phosphorylative activities of heart muscle sarcosomes. Biochem. Jour. 55: 566. 1953.
16. Tager, J. M. The oxidation of pyruvic acid by a particulate fraction from Avena seedlings. Physiol. Plantarum 7: 625–631. 1954.