COFACTOR REQUIREMENTS FOR OXIDATION OF ALPHA-KETO ACIDS BY SWEET POTATO MITOCHONDRIA [1]

M. LIEBERMAN AND J. B. BIALE

BIOLOGICAL SCIENCES BRANCH, U. S. DEPARTMENT OF AGRICULTURE, BELTSVILLE, MARYLAND AND DEPARTMENT OF SUBTROPICAL HORTICULTURE, UNIVERSITY OF CALIFORNIA, LOS ANGELES, CALIFORNIA

In recent years evidence that suggests the operation of the Krebs cycle in plant tissues has accumulated. Studies by Millerd et al (10), Laties (8), Brummond and Burris (4), Biale and Young (3), and Smillie (14), have demonstrated that mitochondria, isolated by differential centrifugation of tissue homogenates, can carry out oxidation with concomitant phosphorylation of Krebs cycle acids. Davies (5), and Abramsky and Biale (1) have shown by chromatographic techniques that the oxidations of Krebs cycle acids are, in fact, mediated through the Krebs cycle and that pyruvate enters the cycle. However, active mitochondria have been isolated from plants of few species. Most of the tissues used for these studies, except those of Biale and Young (3) and Smillie (14), were either juvenile or etiolated. There is, therefore, a need to extend the knowledge of mitochondrial oxidations to many more plant species and to different tissues. The objective of this study was to isolate and characterize mitochondria from sweet potato roots (*Ipomoea batatas* Poir.). It was hoped that work with a different species and a different tissue might throw some additional light on mitochondrial activity in plant cells.

In most metabolic studies with cytoplasmic particles from plants the rates of pyruvate oxidation were considerably lower than those of the other acids. The cofactors used for activating pyruvate oxidase were virtually the same as those for malate, citrate, etc. The requirement for a primer acid, adenylate, and magnesium was established in the early investigations. It seemed desirable, therefore, to explore more fully the relation of some of the less commonly used cofactors to the oxidation of keto acids. This paper reports the results of such studies.

MATERIALS AND METHODS

The sweet potatoes used in these experiments were of the Key West variety. Particulate suspensions were prepared by the differential centrifugation technique as outlined by Biale and Young (3). The roots were chilled at 0° C for 1 hour, peeled, grated, and 200 gm of grated material was homogenized with 300 ml of 0.5 M sucrose in a Waring blendor. Blending was carried out for 2 minutes at 47 volts. The homogenate was centrifuged at 1000 × g for 5 minutes to precipitate starch, whole cells, cell walls, and other large cellular fractions. The supernatant was centrifuged at 14,000 × g for 15 minutes; the precipitated pellet was washed in 20 ml of 0.5 M sucrose and again centrifuged at 14,000 × g for 15 minutes. The resulting precipitate was suspended in 6 ml of 0.5 M sucrose and used as the particulate fraction or the "mitochondrial" preparation. The preparations contained approximately 0.5 mg N per 0.5 ml of suspension. Up to this point all manipulations were carried out at 0° C.

The particles were assayed for activity by standard Warburg manometric techniques at 25° C with air as the gas phase. The volume of the reaction mixture was 3 ml and the solutions added to the vessels are given in the tables and figures or in the appropriate paragraph under "Results."

Nitrogen determinations on the isolated mitochondria were made by Nesslerization of an aliquot of the mitochondrial suspension according to the method of Thompson and Morrison (17) as modified by Biale and Young (3).

In every case the values reported in the tables and figures are averages from at least 3 experiments. Abbreviations used are as follows: $Q_{O_2}(N)$, μl O_2 uptake per mg nitrogen per hour; AMP, adenosine monophosphate; DPN; diphosphopyridine nucleotide; CoA, coenzyme A; and DPT, diphosphothiamine.

RESULTS

OXIDATION OF KREBS CYCLE INTERMEDIATES: The rates of oxidation of several intermediates of the Krebs cycle by the mitochondrial suspension of sweet potato are shown in table I. The oxidation values in table I compare favorably with those reported for other plant tissues for the oxidation of citrate, α-ketoglutarate, and succinate (3, 5, 10). However, it appeared that the ability to oxidize pyruvate was almost totally lacking in sweet potato mitochondria. It, therefore, seemed desirable to study the pyruvate oxidase system in the sweet potato.

COFACTOR REQUIREMENTS FOR PYRUVATE OXIDATION: The general scheme for cofactor cycling in the

TABLE I

OXIDATION OF KREBS CYCLE ACIDS BY SWEET POTATO MITOCHONDRIA *

SUBSTRATE	Q_{O_2} (N)
None	0
Pyruvate **	35
Citrate	216
α-Ketoglutarate	179
Succinate	432

* Reaction mixture consisted of 0.5 M sucrose, 0.001 M AMP, 0.02 M glucose, 0.006 M Mg, 0.01 M P_{O_4}, 0.02 M substrate, and 0.5 ml mitochondrial preparation containing approximately 0.5 mg N, pH 7.1.

** The concentration of malate as a primer was 0.0005 M.

[1] Received April 10, 1956.

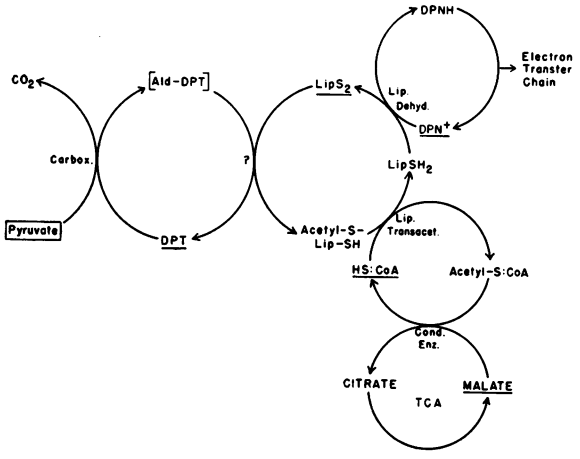

FIG. 1. Scheme for cofactor requirements and cycling in the oxidation of pyruvate [after Gunsalus (7)]. Abbreviations are as follows: Carbox, carboxylase; Ald-DPT, aldehyde-diphosphothiamine complex; Lip-S_2, oxidized lipoic acid; Acetyl-S-Lip-SH, acetyl lipoic complex; Lip-SH_2, reduced lipoic acid; Lip. Dehyd., lipoic dehydrogenase; Lip. Transacet., lipoic transacetylase; HS:CoA, reduced CoA; acetyl-S:CoA, acetyl CoA; Cond. Enz., condensing enzyme. TCA-tricarboxylic acid cycle. The underlined cofactors were selected for this study.

oxidation of pyruvate, as worked out in soluble systems from animal tissues and microorganisms, is presented in figure 1. This diagram, adapted from Gunsalus (7), indicates that DPT, lipoic acid, DPN, CoA, and malate are all involved in pyruvate oxidation.

TABLE II

EFFECTS OF COFACTORS INDIVIDUALLY, IN PAIRS, AND COLLECTIVELY ON OXIDATION OF PYRUVATE BY SWEET POTATO MITOCHONDRIA

COFACTOR* ADDED TO THE BASIC MIXTURE**	Q_{O_2} (N)
None	20
DPN	90
DPT	153
CoA	16
Lipoic acid	12
None	30
DPT	153
DPT + DPN	232
DPT + CoA	119
DPT + lipoic acid	140
None	20
DPN	82
DPN + DPT	334
DPN, DPT + CoA	302
DPN, DPT, CoA + lipoic acid	278

* Concentrations of the cofactors: DPN 3.3×10^{-4} M; DPN 6.6×10^{-5} M, CoA 3.9×10^{-5} M and lipoic acid 3.3×10^{-5} M.

** Content of the basic reaction mixture is given in table I.

On the assumption that these cofactors are similarly concerned in the oxidations by plant mitochondria, it seemed possible that one or more cofactors may have been lost from the mitochondria during their isolation. To test this, systematic additions of cofactors were made to the basic reaction mixture containing the mitochondria and pyruvate as substrate. The basic reaction mixture contained 0.5 M sucrose, 0.01 M PO_4 pH 7.1, 0.001 M AMP, 0.02 M glucose, 0.006 M Mg, 0.0005 M malate as a "sparker," 0.02 M pyruvate, and 0.5 ml of the particulate suspension. To this basic reaction mixture were added singly, doubly, and collectively 3.3×10^{-4} M DPN, 6.6×10^{-5} M DPT, 3.9×10^{-5} M CoA and 3.3×10^{-5} M lipoic acid (kindly supplied by Dr. L. J. Reed of the University of Texas). The results of these studies are shown in table II.

These data (table II) show that good oxidation of pyruvate was obtained only when DPT and DPN were added to the basic reaction mixture. While DPT or DPN alone increased activity markedly, the simultaneous addition of these two cofactors caused an additive increase. The cofactor DPT caused the greater stimulatory effect. On the other hand, neither CoA nor lipoic acid caused any stimulation of activity. This indicated that the addition of these cofactors was not required for the oxidation of pyruvate by isolated sweet potato mitochondria.

TABLE III

MAGNESIUM REQUIREMENT FOR PYRUVATE OXIDATION BY SWEET POTATO MITOCHONDRIA*

MG CONC. $\times 10^{-3}$ M	Q_{O_2} (N)
0	129
0.1	133
1	245
3	247
6	213

* All cofactors added in amounts indicated in table II.

In all the experiments just reported a magnesium concentration of 0.006 M was used as a basic cofactor. However, Young and Biale (18) reported that magnesium concentrations of 0.006 M strongly inhibited pyruvate oxidation by avocado mitochondria. The magnesium requirement for pyruvate oxidation in sweet potato was therefore studied (table III). According to these data magnesium concentrations greater than 10^{-4} M are required in this system for optimal activity. In subsequent experiments a concentration of 10^{-3} M magnesium was added to the cofactor mixture used to mediate pyruvate oxidation.

The importance of adding a primer such as malate in the oxidation of pyruvate has been noted by Millerd (11), Brummond and Burris (4), Tager (16) and others. The purpose of a primer acid is to furnish a condensing partner for acetyl-CoA so that the two-carbon fragment resulting from the oxidative decarboxylation of pyruvate can be introduced into the

TABLE IV

PRIMER ACID REQUIREMENT FOR PYRUVATE OXIDATION BY SWEET POTATO MITOCHONDRIA

COFACTOR ADDITION	MALATE CONC. $\times 10^{-4}$ M	Q_{O_2} (N)
Basic *	0	8
Basic + DPN + DPT	0	12
Basic	5	20
Basic + DPN + DPT	5	302

* For addenda see tables I and II.

Krebs cycle. This reaction is mediated through the condensing exzyme (fig 1), which is presumed to be present in the mitochondria.

Table IV shows that in these systems the priming effect of malate was extremely critical. In the absence of malate there was no oxidation of pyruvate, even though DPN and DPT were present. Conversely, in the presence of the complete basic reaction mixture including malate, there was virtually no oxidation when DPT and DPN were absent.

OXIDATION OF α-KETOGLUTARATE: After obtaining such a striking response to DPT and DPN by the pyruvate oxidase system of sweet potato mitochondria, the importance of these cofactors in the α-ketoglutarate oxidase system was studied. Table V shows the results obtained. The basic reaction mixture contained the same cofactors as for pyruvate oxidation (including 0.006 M magnesium) except that no malate primer was used. It is evident from the data that the collective addition of DPT, DPN, and CoA to the basic reaction mixture considerably increased the oxidation rate of α-ketoglutarate by sweet potato mitochondria. The addition of CoA alone or in combination with DPT caused little increase of the oxidative rate. However, when CoA was added to DPN and DPT there was a marked increase in oxidation rate, indicating a synergistic effect. In this respect the mitochondria reacted differently to the addition of CoA when α-ketoglutarate was oxidized than when pyruvate was oxidized. This may be explained by the considerably higher Q_{O_2}(N) obtained with α-ketoglutarate (approximately 500) than with pyruvate (approximately 300). CoA presumably became limiting at the higher oxidation levels.

TABLE V

EFFECTS OF COFACTORS INDIVIDUALLY, IN PAIRS, AND COLLECTIVELY ON OXIDATION OF α-KETOGLUTARATE BY SWEET POTATO MITOCHONDRIA

COFACTOR ADDED TO THE BASIC MIXTURE *	Q_{O_2} (N)
None	167
DPN	218
DPT	316
CoA	188
Lipoic acid	174
None	178
DPT	316
DPT + DPN	384
DPT + CoA	277
DPT + lipoic acid	249
None	162
DPN	224
DPN + DPT	419
DPN, DPT + CoA	575
DPN, DPT, CoA + lipoic acid	575

* For addenda see tables I and II.

TABLE VI

EFFECTS OF ARSENITE ON OXIDATION OF α-KETO ACIDS *

SUBSTRATE	ARSENITE CONC. $\times 10^{-4}$ M	Q_{O_2} (N)
Pyruvate	0	228
"	5	17
α Ketoglutarate	0	336
"	5	21

* All cofactors added to the reaction mixture (see tables I and II).

ARSENITE INHIBITION: With both α-keto acids there was no response to the addition of lipoic acid. Since arsenite was reported (13) to be a fairly specific inhibitor of enzymes containing active disulfhydryl groups such as lipoic acid, the effect of arsenite in the reaction mixture was studied (table VI). Arsenite completely inhibited the oxidation of pyruvate and α-ketoglutarate, indicating that a lipoic acid like substance is probably present in the sweet potato mitochondria.

CHROMATOGRAPHIC EVIDENCE FOR THE OPERATION OF THE KREBS CYCLE IN THE SWEET POTATO: In order to obtain direct evidence for the formation of Krebs cycle intermediates chromatographic techniques were used to follow the products of the oxidative reactions. The reaction mixture in the Warburg flasks were chromatographed at zero time (immediately after addition of enzyme) and after 120 minutes. After adjustment to pH 5.2, the enzymes were

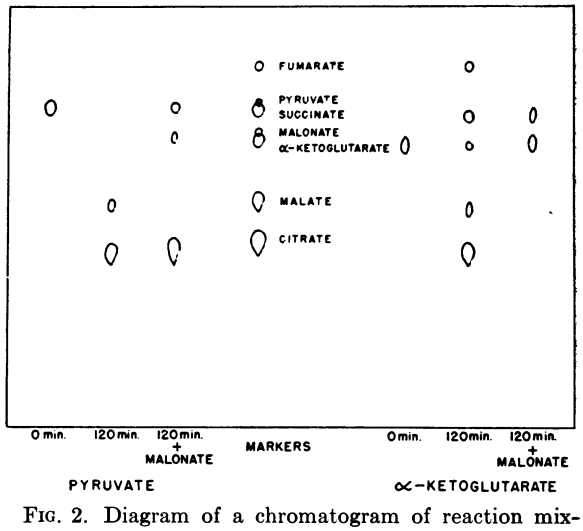

FIG. 2. Diagram of a chromatogram of reaction mixtures containing all cofactors at zero time and after 120 minutes of oxidation.

denatured by heating for 2 minutes, and the mixture was cleared by centrifugation. The clear solution was chromatographed with butanol–5 M formic as the developer and bromphenol blue as the indicator, according to the method of Lugg and Overell (9).

Figure 2 shows one of the chromatograms obtained. At zero time only a spot for the substrate, either pyruvate or α-ketoglutarate, was visible. However, after 120 minutes of reaction in the Warburg vessel the spot for the substrate diminished and other spots, which were identified as intermediates of the Krebs cycle, appeared. When pyruvate was the substrate, spots for citrate and malate appeared. When α-ketoglutarate was the substrate, spots for citrate, malate, succinate and fumarate appeared. In the presence of 0.01 M malonate only succinate was visible on the chromatogram. These data strongly suggest that the oxidations studied were mediated through the Krebs cycle.

Discussion

All the cofactors necessary for oxidation of pyruvate, as outlined in figure 1, are accounted for in these experiments except CoA. However, evidence from the α-ketoglutarate system implies that sufficient CoA is present in the mitochondria for the level of pyruvate oxidation obtained. The cofactor requirements for α-ketoglutarate oxidation are assumed to be essentially the same as for pyruvate except that no priming acid is needed. A requirement for CoA in the α-ketoglutarate system does not become apparent until DPT and DPN are added and the $Q_{O_2}(N)$ is approximately 400. Since the level of pyruvate oxidation is considerably lower with DPT and DPN there is no response to CoA addition. Lipoic acid is presumed to be present in the mitochondria from the data on arsenite inhibition.

Akazawa and Uritani (2) isolated mitochondria from sweet potatoes but did not obtain much activity with any of the Krebs cycle acids as substrates. The data reported herein indicate that an active mitochondrial system can be isolated from sweet potatoes. However, these preparations could not oxidize pyruvate at all and oxidized α-ketoglutarate at a fairly good rate. The addition of suitable cofactors to the reaction mixtures strikingly increased oxidation of both substrates. The assumption is that the basic structure of the isolated mitochondria necessary for these oxidations was intact, since they oxidized the substrate at very good rates after the addition of suitable cofactors.

The absence of cofactors from the isolated mitochondria can be explained in one of two ways. It can be assumed that the isolating procedure removes or destroys the cofactors from the mitochondria by solubilization, by extraction, or by other means, without altering their basic structure. The function of the mitochondria, therefore, appears reversible with respect to these cofactors. It is possible that the enzymes in question are either on the surface of the mitochondria or in the mitochondrial membrane or are in some way made easily accessible to added cofactors. The electron microscope photographs of Palade (12) and Sjostrand (15) show mitochondrial membranes that may well contain enzymes. Apparently lipoic acid is rather firmly attached and remains within the mitochondrial framework after isolation. It is assumed that in vivo the cofactors are directly associated with their enzymes in the mitochondria. Another possible explanation is that the cofactors are not present in the mitochondria, in vivo, but are in the cytoplasmic environment surrounding the mitochondria in the cell. In this arrangement there must be rapid and complete interaction between the enzymes located in the mitochondria and their coenzymes in the surrounding cytoplasmic medium. This concept is not in agreement with Green's (6) picture of the mitochondria as independent integrated enzyme systems (the cyclophorase) containing all the necessary cofactors within their structure.

Summary

Mitochondria isolated from sweet potatoes oxidized most Krebs cycle acids in the presence of AMP, Mg, glucose, and phosphate, but could not oxidize pyruvate. The addition of the cofactors DPT and DPN to the reaction mixture brought about high rates of pyruvate oxidation. The oxidation of α-ketoglutarate was increased more than 200 % by addition of DPT, DPN, and CoA. Exogenous lipoic acid had no effect with pyruvate or α-ketoglutarate as substrate. However, arsenite completely inhibited the oxidation of both α-keto acids indicating the possible presence of lipoic acid in the mitochondria. Chromatographic evidence suggests that the oxidation of the α-keto acids by these mitochondria is mediated through the Krebs cycle.

Since the preparation of these manuscripts the articles by Beevers, H., and Walker, D. A. (Biochem. Jour. 62: 114–119. 1956) and by Walker, D. A., and Beevers, H. (Biochem. Jour. 62: 120–127. 1956) appeared. They used the same cofactors and arrived at substantially the same results with castor bean seedlings as we did with the sweet potato.

The authors wish to express their appreciation for the technical assistance of Harold Neimark.

Literature Cited

1. Abramsky, M. and Biale, J. B. The pyruvate oxidation system in avocado fruit particles. Abstr., Plant Physiol., 30: xxviii–xxix. 1955.
2. Akazawa, T. and Uritani, I. Respiratory oxidation and oxidative phosphorylation by cytoplasmic particles of sweetpotato. Jour. Biochem. (Japan) 41: 631–638. 1954.
3. Biale, J. B. and Young, R. E. Oxidative phosphorylation in relation to ripening of the avocado fruit. Abstr. 13, Amer. Soc. Plant Physiol. Western Sect. Ann. Meeting Santa Barbara, California, June 16 to 18, 1953.

4. BRUMMOND, D. O. and BURRIS, R. H. Transfer of C^{14} by lupine mitochondria through reactions of the tricarboxylic acid cycle. Proc. Natl. Acad. Sci. (U.S.) 39: 754–759. 1953.
5. DAVIES, D. D. The Krebs cycle enzyme system of pea seedlings. Jour. Exptl. Bot. 4: 173–183. 1953.
6. GREEN, D. E. The cyclophorase system. In: Enzymes and Enzyme Systems, Their State in Nature. Memoirs, Univ. Lab. Phys. Chem., Harvard University. No. 1. Harvard University Press, Cambridge, Massachusetts. 1951.
7. GUNSALUS, I. C. Group transfer and acyl generating function of lipoic acid derivatives. Symposium on Mechanism of Enzyme Action, McCollum Pratt Institute, Baltimore, Maryland. 1954.
8. LATIES, G. G. Dual role of adenylate in the mitochondrial oxidations of higher plants. Physiol. Plantarum 6: 199–214. 1953.
9. LUGG, J. W. H. and OVERELL, B. T. One and two dimensional partition chromatographic separations of organic acids on an inert sheet support. Australian Jour. Sci. Research A. 1: 98–111. 1948.
10. MILLERD, A., BONNER, J., AXELROD, B., and BANDURSKI, R. Oxidative and phosphorylative activity of plant mitochondria. Proc. Natl. Acad. Sci. (U.S.) 37: 855–862. 1951.
11. MILLERD, A. Respiratory oxidation of pyruvate by plant mitochondria. Arch. Biochem. Biophys. 42: 149–163. 1953.
12. PALADE, G. E. The fine structure of mitochondria. Anatomical Record 114: 427–451. 1952.
13. PETERS, R. A. The study of enzymes in relation to selective toxicity in animal tissues. Symposia Soc. Exptl. Biol. 3: 36–59. 1949.
14. SMILLIE, R. M. Enzymatic activity of particles isolated from various tissues of the pea plant. Australian Jour. Biol. Sci. 8: 186–195. 1955.
15. SJOSTRAND, F. S. Electron microscopy of mitochondria and cytoplasmic double membranes. Nature 171: 30–32. 1953.
16. TAGER, J. M. The oxidation of pyruvic acid by a particulate fraction from Avena seedlings. Physiol. Plantarum 7: 625–636. 1954.
17. THOMPSON, J. F. and MORRISON, G. P. Determination of organic nitrogen. Control of variables in the use of Nessler's reagent. Anal. Chem. 23: 1153–1157. 1951.
18. YOUNG, R. E. and BIALE, J. B. Effects of EDTA on oxidations mediated by avocado mitochondria. Abstr., Amer. Soc. Plant Physiol. Western Sect. Ann. Meeting Pullman, Washington, June 22 to 24, 1954.

FLORAL INITIATION IN CESTRUM NOCTURNUM, A LONG-SHORT DAY PLANT. II. A 24-HOUR VERSUS A 16-HOUR PHOTOPERIOD FOR LONG DAY INDUCTION[1]

ROY M. SACHS[2,3]

EARHART PLANT RESEARCH LABORATORY, DIVISION OF BIOLOGY, CALIFORNIA INSTITUTE OF TECHNOLOGY, PASADENA, CALIFORNIA

Recent experiments have shown that *Cestrum nocturnum* is a long-short day plant, requiring first long day and then short day induction (5). The purpose of this paper is to make some comparisons between long day induction in Cestrum and in simple long day plants.

METHODS, RESULTS, AND DISCUSSION

The experiments were performed in the Earhart Laboratory (6) using the 26° C greenhouse and 20° C temperature controlled rooms (for both artificial light and darkness). The light intensity was generally in excess of 1500 ft-c in the greenhouse and approximately 700 ft-c at plant tops in the artificial light rooms in which the light was supplied by warm, white fluorescent tubes supplemented with incandescent bulbs.

All of the plants used were grown from seed and were more than 3 months old at the beginning of long day induction. The criteria for the selection of plants and quantitative measurement of floral initiation were the same as those described previously (5). The errors quoted for the number of nodes flowering per plant are the standard errors of the means; the "t" test (3) was applied assuming that the samples were taken from normal distributions.

Two groups of plants were exposed to 12 or 18 long days with a 16-hour photoperiod (8 hours in the greenhouse plus 8 hours of artificial light and 8 hours of darkness). Two other lots received 12 or 18 long days with a 24-hour photoperiod (8 hours in the greenhouse plus 16 hours of artificial light). At the end of the respective long day treatments the plants were transferred to short day conditions (8 hours in the greenhouse plus 16 hours of darkness) for 7 days. The results of the experiment, recorded in table I, show that for 12 days a 16-hour photoperiod was greatly superior to a 24-hour photoperiod for long day induction. As the number of long days was increased the difference between the two treatments decreased. This experiment was repeated twice with essentially the same results. Apparently in the 8 hours of darkness between the end of one 16-hour photocycle and the beginning of another, a reaction(s) may be activated by light which reduces the effectiveness of long day induction.

It has been reported for long day plants that continuous light (24-hour photoperiod) is the most

[1] Received April 24, 1956.

[2] Received financial aid from the Arthur H. McCallum and Lucy Mason Clark Fellowship Funds and a U. S. Government Grant under the Fulbright Act.

[3] Present address: Department of Botany, University of California, Los Angeles 24, California.