TRANSFER OF C^{14} BY LUPINE MITOCHONDRIA THROUGH REACTIONS OF THE TRICARBOXYLIC ACID CYCLE*

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Many observations of the behavior of organic acids in plants are explicable in terms of the functioning of a tricarboxylic acid cycle. Generally the most abundant organic acids in plant tissues are malic, citric, and oxalic acids. Oxalate is relatively inert and appears to be an end-product rather than an active metabolite. As malate and citrate show marked quantitative responses to changes in the physical environment of the plant and behave otherwise as active metabolites, it has been proposed that several of their conversions are mediated through a tricarboxylic acid cycle. For example, Pucher, Wakeman, and Vickery[1] have shown that excised tobacco leaves in the dark lose malate and accumulate citrate, and Zbinovsky and Burris[2] have found that tobacco leaves infiltrated with C^{14}-labeled malate produce labeled citrate.

Within the past few years, evidence has accumulated in support of the existence of the tricarboxylic acid cycle in plants, and the recent observations of Millerd *et al.*,[3, 4] and Bonner and Millerd[5] compel one to conclude that the cycle is operative in certain plant tissues. These workers have demonstrated the oxidation of the acids of the tricarboxylic acid cycle and the oxidative phosphorylation by mitochondria from mung bean seedlings. In the experiments reported here, the presence of the cycle is indicated by transfer of radiocarbon from pyruvate-2-C^{14} into the intermediates of the tricarboxylic acid cycle.

Methods and Materials.—Preparations of particles, identified as mitochondria by morphology and affinity for janus green, were obtained from the cotyledons of etiolated white lupine seedlings in a manner similar to that described by Millerd *et al.*[3] The seeds (*Lupinus albus*) were soaked for 12 hours in distilled water prior to incubation in moist sand at 28°C. The cotyledons of seven-day-old seedlings were removed, and 70 g. of cotyledons, 70 ml. of 0.2 *M* sucrose solution and enough solid K_2CO_3 to bring the final pH of the brei to 6.5–7.0 were ground together with quartz sand in a mortar. All operations preceding the measurement of activity were performed at 0°C. and solutions and utensils were prechilled to this temperature. The brei was strained through cheese cloth and centrifuged at 1000× G for 10 minutes to remove nuclei and cell debris. The supernatant was then centrifuged at 20,000× G for 10 minutes, and the yellow pellet obtained was resuspended in 0.2 *M* sucrose and recentrifuged at 20,000× G for 15 minutes. This yellow sediment resuspended in 20–25

ml. of 0.2 *M* sucrose was used as the enzyme preparation. The suspension contained 0.5–1.0 mg. N/ml. and constituted half the volume of the reactants in the manometric flask. All of the experiments were performed at 30°C. in conventional Warburg manometric apparatus.

Pyruvate, oxalacetate, α-ketoglutarate, and oxalosuccinate were separated as their 2,4-dinitrophenylhydrazones on silica gel columns by a method similar to that employed by Sanger[6] for the separation of the 2, 4-dinitro-N-phenyl derivatives of amino acids. The columns were prepared by uniformly mixing 1.4 ml. of an aqueous buffer solution, which was equilibrated against the organic solvent used for developing the column, with 2.0 g. of silica gel (Mallinckrodt, 100 mesh, analytical reagent silicic acid). About 25 ml. of the organic phase was mixed with the silica gel, and the resulting slurry was poured into a 1 cm. diameter column. The solvent systems used were 5 per cent butanol, 95 per cent chloroform (all solvents given as volume per cent) equilibrated against a 1.0 *M* potassium phosphate buffer of pH 6.8; 15 per cent butanol, 85 per cent chloroform equilibrated against a 1.0 *M* potassium formate buffer of pH 3.8; and 7 per cent butanol, 93 per cent chloroform equilibrated against a 1.0 *M* potassium phosphate buffer of pH 2.0.

The hydrazones of the keto acids were prepared by mixing an excess of a 0.1 per cent solution of 2,4-dinitrophenylhydrazine in 2.0 *N* HCl with the free acids or with the preparation to be determined according to the method of LePage.[7] The hydrazones then were extracted with ether in a Kutscher-Steudel extractor for 1 hour; the ether was removed under vacuum, and the residue was taken up in 0.5–1.0 ml. of the organic developing solvent and was placed on the appropriate column. For separation of the oxalosuccinate derivative, the 7 per cent butanol, 93 per cent chloroform solvent was employed. Derivatives of oxalacetate and α-ketoglutarate were separated with the 15 per cent butanol, 85 per cent chloroform solvent, and the pyruvate 2,4-dinitrophenylhydrazone was recovered with 95 per cent chloroform, 5 per cent butanol as solvent. In these systems, the pyruvate and oxalacetate 2,4-dinitrophenylhydrazones were resolved into their geometric isomers. Recoveries of 98–101 per cent were obtained from known pyruvate, oxalacetate, and α-ketoglutarate when about 100 μg. quantities of these acids were determined. The quantity of each 2,4-dinitrophenylhydrazone was measured in a 1 cm. cell in the solvent in which it was eluted from the column; the quantitative observations were made in a Beckman model DU spectrophotometer at the wave length of maximum absorption for each derivative (range 340 to 365 mμ).

Experimental Results and Discussion.—As shown in figure 1, the addition of magnesium ions and ATP (adenosine triphosphate) stimulated the oxidation of pyruvate plus malate by mitochondria, but Armour Labora-

tories' "liver coenzyme concentrate" stimulated much more. Figure 2 indicates that the addition of DPN of greater than 95 per cent purity stimulated the oxidation of malate plus pyruvate. Armour Laboratories' "liver coenzyme concentrate," which contains DPN, TPN (di- and triphosphopyridine nucleotides) and CoA (coenzyme A) and which stimulated the uptake of oxygen, was added routinely in later experiments.

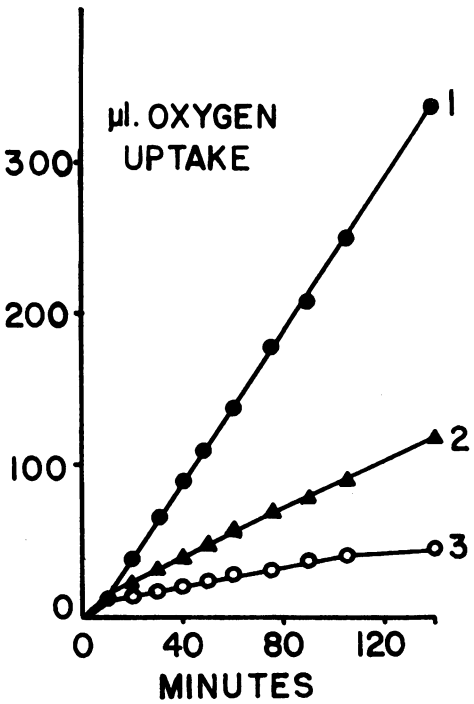

FIGURE 1

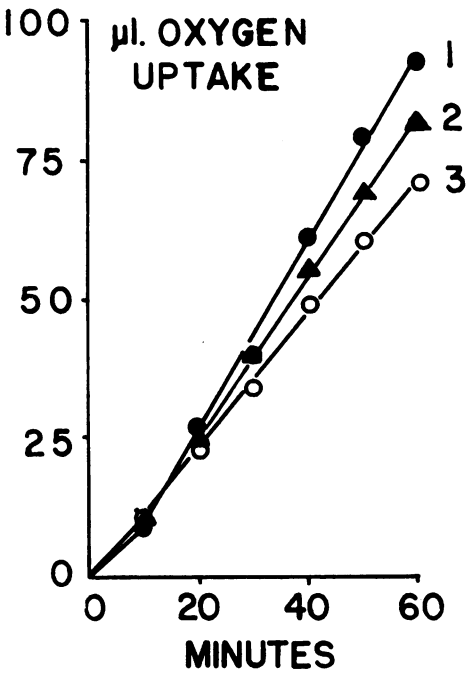

FIGURE 2

Fig. 1—The influence of ATP (adenosine triphosphate) plus $MgSO_4$, and of CoA (coenzyme A) on the oxidation of pyruvate plus malate by lupine mitochondria. Each flask contained mitochondria, 50 µm potassium phosphate buffer pH 7.0, 20 µm L-malate, and 20 µm pyruvate; final volume 2.0 ml. Temperature, 30°C. (*1*) 1 mg. "liver coenzyme concentrate" added. (*2*) 2 µm $MgSO_4$ plus 0.2 µm ATP. (*3*) Nothing added. Fig. 2—The influence of diphosphopyridine nucleotide and coenzyme A on the oxidation of pyruvate plus malate by lupine mitochondria. Substrates and buffers supplied as in figure 1. (*1*) 2.5 mg. "liver coenzyme concentrate" added. (*2*) 0.25 or 0.05 mg. DPN added. (*3*) 0.25 mg. "liver coenzyme concentrate" added.

In contrast to the preparations of Millerd *et al.*,[3] it was unnecessary to grind the lupine tissue in phosphate buffer; added cytochrome-c did not influence the uptake of oxygen. The rates of oxidation of pyruvate in the presence of L-malate or oxalacetate are shown in figure 3. As oxalacetate inhibited oxygen uptake for the first 30 minutes, L-malate was used subsequently as the "sparking" acid.

Manometric measurements indicated that malate and pyruvate in combination were oxidized at a more rapid rate by the mitochondrial preparations than the sum of their rates singly. These preparations thus seemed to provide an enzymatic system suitable for testing incorporation of a tracer into intermediates of the tricarboxylic acid cycle. Tests were made by adding to a mitochondrial preparation 200 μm of pyruvate-2-C^{14}, 200 μm of L-malate and 200 μm of another acid of the cycle to serve as a trapping acid. During an hour's incubation at 30°C. in a 125-ml. Warburg flask, the trapping acid equilibrated with the same acid as it was formed enzymatically. Then the trapping acid and any other acids present in sufficient quantity were isolated by partition chromotography on silica gel[8] and were analyzed for their content of C^{14}.

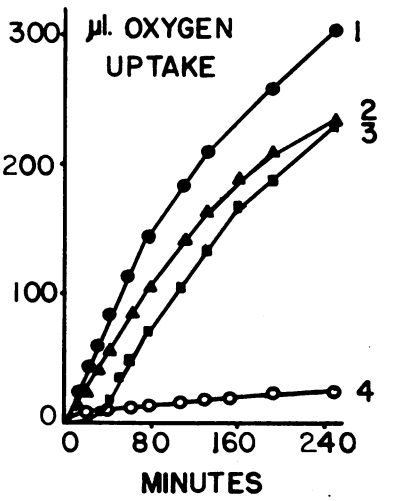

FIGURE 3

The effect of malate and oxalacetate on the oxidation of pyruvate by lupine mitochrondria. All flasks contained 50 μm potassium phosphate buffer pH 7.0, 1 mg. "liver coenzyme concentrate" and mitochondria in a final volume of 2.0 ml. (*1*) 10 μm malate plus 20 μm pyruvate. (*2*) 10 μm malate. (*3*) 10 μm oxalacetate plus 20 μm pyruvate. (*4*) 10 μm oxalacetate or 20 μm pyruvate alone.

Data presented in table 1 show that there is an appreciable incorporation of C^{14} from pyruvate-2-C^{14} into every acid of the tricarboxylic acid cycle; this indicates the participation of each acid in the cyclic oxidation of pyruvate. In the experiment in which malate was supplied, there was a net accumulation of 21 μm of citrate. The specific activity of the citrate was 32.6 counts $\times$ min.$^{-1}$ $\times$ μm^{-1} and that of the original pyruvate 31.5 counts $\times$ min.$^{-1}$ $\times$ μm^{-1}. Thus it is evident that the citrate arose directly from pyruvate and malate, since the molar specific activities of citrate and pyruvate were essentially the same. The substantial accumulation of citrate suggests the presence of an active condensing enzyme which can effect a condensation of acetyl CoA and oxalacetate as occurs in isolated animal tissues.[9] That the subsequent oxidation of pyruvate proceeds via the other acids of the tricarboxylic acid cycle is shown by the accumulation of appreciable amounts of C^{14} into every acid of the cycle. The variability of the amount of C^{14} found in individual acids after each experiment probably resulted from variability in recovery as well as differences in the activity of specific preparations. The accumulation of C^{14} in the respired CO_2 shows that the oxidation of a portion of the

pyruvate is complete. The small percentage accumulation of C^{14} in the respired CO_2 in these experiments can be attributed to the effective trapping of the C^{14} by the pools of acids added as sparker and trapping agents. This small accumulation of $C^{14}O_2$ also constitutes evidence for the asymmetrical synthesis and oxidation of citric acid in plant tissues, a process previously demonstrated in animal tissue.[10] As much C^{14} should have appeared in the CO_2 as appeared in succinate, fumarate, malate, and oxalacetate if the conversion had been symmetrical.

These results, in conjunction with earlier evidence that mitochondria can readily oxidize the intermediates of the tricarboxylic acid cycle, strongly support the premise that pyruvate oxidation by lupine mitocondria proceeds via the conventional tricarboxylic acid cycle. As plants can readily form pyruvate from carbohydrates, it is evident that carbohydrates in plants can be completely oxidized to CO_2 and H_2O in a manner analogous to that of the animal cell. How widespread and active the tricarboxylic acid cycle is in plants and whether it is functional in mature plant tissues remain to be established.

TABLE 1

THE ACCUMULATION OF C^{14} IN CO_2 AND ACIDS OF THE TRICARBOXYLIC ACID CYCLE FROM PYRUVATE-2-C^{14} SUPPLIED TO PREPARATIONS OF LUPINE MITOCHONDRIA

TRAPPING ACID ADDED	PER CENT C^{14} FOUND IN TRAPPING ACID RECOVERED	PER CENT C^{14} FOUND IN CO_2 FORMED	PER CENT C^{14} FOUND IN CITRATE FORMED	PER CENT C^{14} FOUND IN MALATE RECOVERED
Citrate	6.5	1.0	..	0.7
Cis-aconitate	1.5	0.5	30.2	0.6
Isocitrate	2.2	1.0	49.0	5.0
Oxalosuccinate (30 min. reaction)	0.5	1.5	..	...
α-Ketoglutarate	7.5	1.0	..	...
Succinate	5.5	1.0	..	...
Fumarate	4.4	1.5	..	...
Malate	2.0	1.0	10.9	...
Oxalacetate	2.3	1.5	..	...
Pyruvate alone	...	0.2	..	...

Summary.—Methods for the chromatographic separation and determination of the 2,4-dinitrophenylhydrazones of pyruvate, α-ketoglutarate, oxalacetate, and oxalosuccinate are described. Recoveries greater than 98 per cent for 100 μg. quantities of pyruvate, α-ketoglutarate, and oxalacetate are obtained; the range for each acid is 10–500 μg.

Mitochondria isolated from the cotyledons of etiolated lupine seedlings oxidize pyruvate via the tricarboxylic acid cycle in a manner analogous to that of animal mitochondria. Individual experiments in which pyruvate-2-C^{14} plus unlabeled malate were oxidized by lupine mitochondria in the presence of one other acid of the tricarboxylic acid cycle demonstrated incorporation of C^{14} into each acid of the cycle so tested.

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[1] Pucher, G W., Wakeman, A. J., and Vickery, H. B., *J. Biol. Chem.*, **119**, 523–534 (1937).

[2] Zbinovsky, V., and Burris, R. H., *Plant Physiol.*, **27**, 240–250 (1952).

[3] Millerd, A., Bonner, J., Axelrod, B., and Bandurski, R., PROC. NATL. ACAD. SCI., **37**, 855–862 (1951).

[4] Millerd, A., *Arch. Biochem. Biophys.*, **42**, 149–163 (1953).

[5] Bonner, J., and Millerd, A., *Ibid.*, **42**, 135–148 (1953).

[6] Sanger, F., *Biochem. J.*, **39**, 507–515 (1945).

[7] LePage, G. A., *Cancer Research*, **10**, 393–397 (1950).

[8] Isherwood, F. A., *Biochem. J.*, **40**, 688–695 (1946).

[9] Korkes, S., del Campillo, A., Gunsalus, I. C., and Ochoa, S., *J. Biol. Chem.*, **193**, 721–735 (1951).

[10] Wilcox, P. E., Heidelberger, C., and Potter, V. R., *J. Am. Chem. Soc.*, **72**, 5019–5024 (1950).

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*SUBCELLULAR FRACTIONATION OF MOUSE SPLEEN RADIATION PROTECTION ACTIVITY**

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Jacobson, *et al.*[1] have demonstrated that the survival of mice exposed to lethal doses of x-rays can be increased significantly by implantation of normal mouse spleens into the peritoneal cavity of irradiated mice after exposure. These findings have led to the concept of a humoral radiation recovery factor (or factors) present in, and elaborated by, normal mouse spleen tissue.[2] They were followed by experimental studies from this Laboratory,[3, 4] in which it was shown that the intraperitoneal injection of neutral phosphate buffer homogenates of normal mouse spleen into mice which had received otherwise lethal whole body x-irradiation, afforded marked protection against mortality. These studies provided a reproducible experimental procedure for obtaining active, injectable spleen preparations in which the vast majority of the cells had been disrupted, and in which the level of organization was far removed from that of the original spleen tissue. Furthermore, the experimental procedure provided a suitable biological assay for testing the protective activity of various spleen