of the mutant gene. It was suggested that the gene may be operating on a common pathway for the synthesis of the chlorophylls and carotenoids, at a position after the synthesis of carotenes, but before the synthesis of the carotenols and the phytyl portion of the chlorophyll molecule.

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OXIDATIVE PHOSPHORYLATION BY SWEET POTATO MITOCHONDRIA AND ITS INHIBITION BY POLYPHENOLS [1]

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In a previous study (11) it was found that the rate of oxidation of α-ketoglutarate by a mitochondrial preparation from sweet potato roots was considerably enhanced when diphosphothiamine, diphosphopyridine nucleotide, and coenzyme A were added to a basic reaction mixture of adenylate, magnesium, and glucose in a phosphate buffer at pH 7.0. It was of interest, therefore, to examine the phosphorylative capacity of this reconstituted α-ketoglutarate oxidase. The specific purpose of this paper is to report the conditions that have been found for optimum oxidative phosphorylation and the effects of some inhibitors on oxygen and phosphorus uptakes by the particulate fraction of the sweet potato.

Materials and Methods

Key West sweet potatoes (*Ipomoea batatas* Poir) were used in these experiments. The mitochondria were prepared by the methods outlined in another paper (11). The final mitochondrial pellet was suspended in 10 ml of 0.5 M sucrose instead of 6 ml as used for the studies on cofactor requirements. Assay of oxidative activity by Warburg techniques and nitrogen determinations were carried out as already described (11). Inorganic phosphorus was determined by the method of Bernhart and Wreath (2). Each experiment was conducted at least 3 times and the data reported are averages from these experiments.

Results

Effects of Phosphate and Hexokinase on Oxidative Phosphorylation: Rates of oxidation and phosphorylation were studied in increasing concentrations of phosphate in the presence and absence of hexokinase. Phosphorus was determined immediately after the addition of the mitochondria to

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the reaction mixture (zero-time) by rapidly withdrawing a 1-ml aliquot of the reaction mixture and pipetting into cold 1 M trichloroacetic acid (TCA). A second aliquot was removed and treated similarly after 30 minutes at 25° C. The denatured enzymes were precipitated by centrifugation, and an aliquot of the supernatant was assayed for inorganic phosphorus. The difference in phosphorus content between the "30-minute" and the "zero-time" samples was ascribed to the incorporation of inorganic phosphorus into the organic form.

Phosphate concentrations of 30 micromoles per 3 ml resulted in high oxygen uptake irrespective of the presence of hexokinase (fig 1). With higher concentrations of phosphate little or no further increase in oxygen uptake was noted. The presence of hexokinase caused a significant increase in oxidation at the two lowest phosphate levels. This indicates that

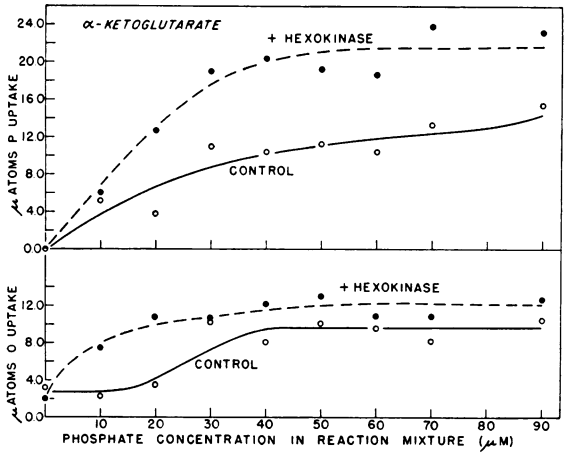

FIG. 1. Oxidative phosphorylation in the α-ketoglutarate system at different levels of phosphate in presence and absence of hexokinase (30-minuate experiment).

Concentration of phosphate is in μM/3 ml.

at low phosphate levels the uptake of oxygen is more closely coupled to the phosphorylating system.

Phosphorus uptake was considerably increased with increase in phosphate concentrations in the range of 0 to 30 micromoles in the medium. However, as the concentration of phosphate was increased from 30 to 90 micromoles, there was little further increase in phosphorus uptake. The actual quantities of phosphorus incorporated into organic form in the 30 to 90 micromole range were approximately 75 to 100 % greater in systems containing hexokinase than in the controls. There was much greater response to hexokinase in phosphorus uptake than in oxygen uptake. This can be explained on the basis of higher phosphorylative efficiency in the presence of hexokinase resulting perhaps in less loss due to phosphatase.

EFFECT OF ETHYLENEDIAMINE TETRAACETIC ACID (EDTA) IN THE PREPARATIVE MEDIUM: It was reported that the chelating agent EDTA stimulated the oxidative activity of mitochondria obtained from

TABLE I

COMPARISON OF OXIDATION AND PHOSPHORYLATION BY SWEET POTATO MITOCHRONDRIA *

PREPARATION MEDIUM	MALONATE	P UPTAKE	O UPTAKE	P/O
	M	*μ atoms*	*μ atoms*	
Sucrose (0.5 M)	0	23.7	11.8	2.0
" + EDTA (0.01 M)	0	36.0	16.8	2.2
Sucrose (0.5 M)	0.01	15.1	5.7	2.7
" + EDTA (0.01 M)	0.01	29.6	8.9	3.3

* The reaction mixture contained: 0.5 M sucrose, 0.02 M PO_4, 0.001 M AMP, 0.02 M glucose, 0.006 M Mg, 0.02 M α-ketoglutarate, 1 mg hexokinase, 3.3×10^{-4} M DPN, 6.6×10^{-5} M DPT, 3.9×10^{-5} M CoA, 0.5 ml mitochondrial suspension containing approximately 0.5 mg protein N; total volume 3 ml; pH 7.1; temp. 25° C; gas phase, air.

Avena seedlings (17) and from broccoli buds (12) and stabilized oxidative phosphorylation mediated by heart muscle sacrosomes (16). Hence the activity of mitochondria prepared in EDTA-sucrose was compared with that in sucrose alone. It can readily be seen (table I) that preparation in EDTA-sucrose considerably increased both oxygen and phosphorus uptake. Phosphorus uptake was increased approximately 50 % in treatments without malonate and approximately 100 % in the malonate treatments. Oxygen uptake was increased approximately 50 % in both treatments.

On the basis of these data all subsequent mitochondrial preparations were homogenized in 0.5 M sucrose + 0.01 M EDTA. In the reaction mixture the phosphate content was 60 micromoles for experiments of 30 to 40 minutes' duration and 90 micromoles for experiments of 1 hour's duration. In all cases 1 mg of hexokinase (Pabst) was also supplied to each vessel.

RATE OF OXIDATIVE PHOSPHORYLATION: The purpose of the next series of experiments was to determine the time course of phosphorus and oxygen uptake during 1 hour's oxidation under optimum con-

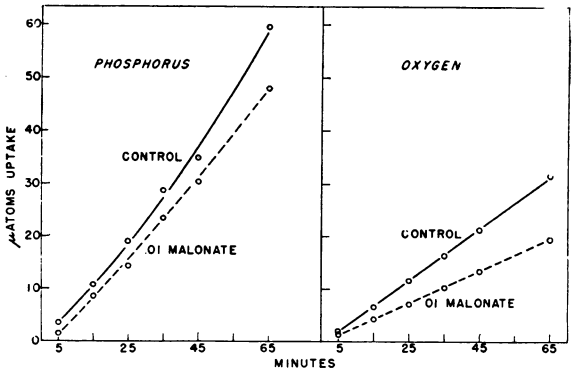

FIG. 2. Rate of oxidation and phosphorylation in the α-ketoglutarate system by sweet potato mitochondria in presence and absence of malonate.

ditions. Presumably if the system were complete the rates of oxidative phosphorylation would be reasonably constant for a considerable period of time. Malonate was added to half of the experimental flasks to limit the oxidation essentially to the single step, α-ketoglutarate to succinate.

From the data in figure 2 it is evident that oxidation and phosphorylation continued at constant rates during a 65-minute period, both in the presence and in the absence of malonate. Malonate inhibited phosphorylation by approximately 15 % and oxygen uptake by approximately 30 %. In the absence of malonate approximately 60 micromoles of phosphorus were incorporated during 1 hour of oxidation. The P/O ratios were approximately 2 in the absence of malonate and approximately 2.5 in its presence.

Effect of Dinitrophenol (DNP): The effect of DNP as a uncoupler of oxidative phosphorylation has been studied by many workers in both animal and

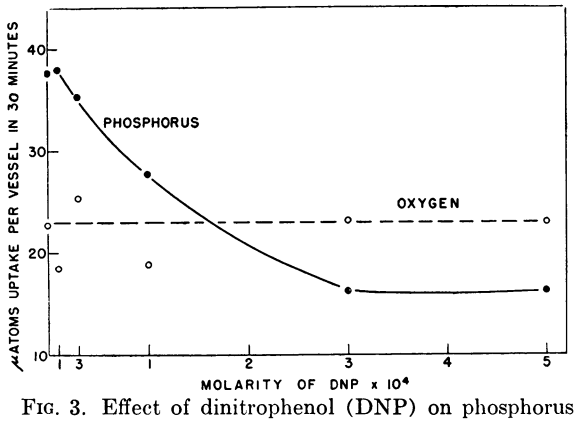

Fig. 3. Effect of dinitrophenol (DNP) on phosphorus and oxygen uptake during 30-minute oxidation of α-ketoglutarate.

plant systems (3, 4, 7, 9). It was of interest to see whether the classical effect of this substance could be demonstrated in the sweet potato mitochondrial system. The effect of various concentrations of DNP on oxygen and phosphorus uptake during the oxidation of α-ketoglutarate in these systems is shown in figure 3.

At all the concentrations tried, which included the range from 1×10^{-5} M to 5×10^{-4} M, there was apparently little consistent change in oxygen uptake as compared with that in the control. A DNP concentration of 1×10^{-4} M inhibited phosphorus uptake about 25 %, while concentrations of 3×10^{-4} M, and 5×10^{-4} M DNP inhibited it about 60 %.

If DNP inhibits all the phosphorylation occurring in the electron transport chain (9), the phosphorus uptake of 17 micro-atoms at 3×10^{-4} M DNP may be considered as substrate-level phosphorylation plus whatever had been lost by phosphatase activity. On this basis the total theoretical phosphorus uptake in the control should be 68+ micro-atoms since the theoretical P/O ratio is 4. However, the experimental value of phosphorus uptake in the control was only 38 micro-atoms with a P/O ratio of 1.7. Apparently part of the electron-transport chain was not phosphorylating. The DNP concentrations effective in inhibiting phosphorylation did not seem to cause concomitant increase in oxygen uptake. On the other hand, when the rates were calculated for different intervals during the experiment, DNP did cause accelerated oxygen uptake, as can be seen in table II. After 20 minutes, the two concentrations that most effectively inhibited phosphorus uptake also markedly accelerated oxygen uptake. Since there was considerable inhibition of oxygen uptake during the first 10 minutes, in the presence of DNP, the true picture of oxygen uptake is distorted if one considers total uptake during a 30-minute experiment. The accelerating effect of DNP on oxidation was more readily seen when the time course of oxygen uptake, in the presence of 3×10^{-4} M DNP, was studied during a 60-minute experiment (fig. 4).

Table II

Effect of DNP on Rate of Oxygen Uptake during 30-Minute Oxidation *

Conc. DNP	Rate of oxygen uptake		
	5–10 Min	10–20 Min	20–30 Min
$M \times 10^{-4}$	$\mu l/hr$ (*extrapolated*)		
0	444	432	438
5	264	450	546
3	252	456	606
1	336	408	390
0.1	372	348	384

* Reaction mixture same as in table I.

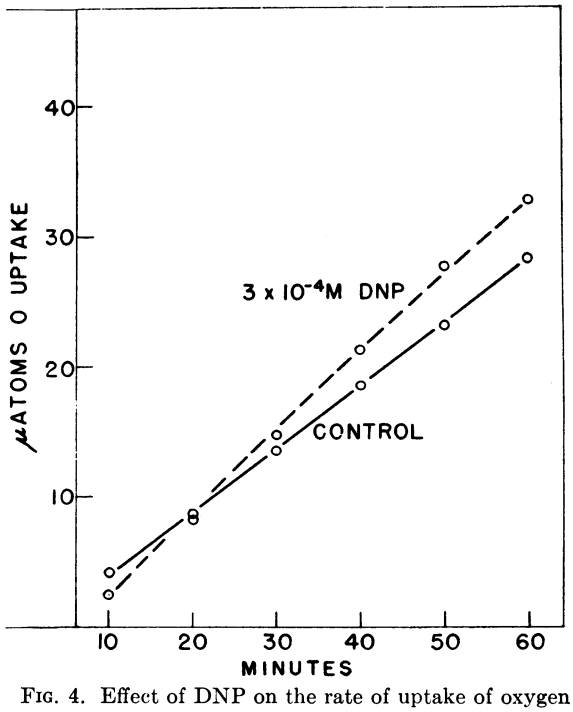

Fig. 4. Effect of DNP on the rate of uptake of oxygen during the oxidation of α-ketoglutarate.

TABLE III

EFFECT OF VARIOUS CONCENTRATIONS OF CATECHOL ON OXIDATION AND PHOSPHORYLATION IN THE α-KETOGLUTARATE SYSTEM

CONC. CATECHOL	O UPTAKE	P UPTAKE	P/O
$M \times 10^{-5}$	μ atoms	μ atoms	
0*	28.6	49.7	1.7
1	23.0	46.5	2.0
3	27.0	49.0	1.8
10	3.2	0.0	0.0
30	1.6	0.0	0.0
60	0.0	0.0	0.0

* Reaction mixture same as in table I.

EFFECT OF POLYPHENOLS ON OXIDATIVE PHOSPHORYLATION DURING THE OXIDATION OF α-KETOGLUTARATE: In view of the pronounced effect of DNP in uncoupling oxidation from phosphorylation, it had been postulated (13) that natural substances in the cell may function similarly as uncouplers, especially during senescence. Akazawa and Uritani (1) reported that chlorogenic acid, a natural polyphenolic substance in sweet potatoes (15), uncoupled oxidation from phosphorylation during the oxidation of a Krebs cycle intermediate. We decided, therefore, to use catechol, a simple polyphenolic substance, to test the effect of polyphenols on the oxidation of α-ketoglutarate by the sweet potato mitochondria in the complete system.

Concentrations of catechol up to 3×10^{-5} M had no effect on oxidation or phosphorylation (table III). However, concentrations of 10^{-4} M and higher almost completely inhibited both oxidation and phosphorylation. Since catechol is very easily oxidized to the quinone form it seemed plausible to assume that oxidation of catechol occurred and was related to the inhibition of oxidative phosphorylation in these systems. The mitochondria exhibited some polyphenolase activity indicating that catechol could be oxidized by the mitochondria.

When ascorbic acid, which can keep the polyphenol in the reduced state, was added to the complete α-ketoglutarate system containing catechol, the inhibitory effect was prevented (table IV). Substitution of chlorogenic acid for catechol in these experiments gave identical results (table IV). From these data it was concluded that the oxidized polyphenols (quinones) or their polymerization products strongly inhibited oxidative phosphorylation in the α-ketoglutarate system and that the reduced form of the polyphenol was innocuous.

TABLE IV

EFFECT OF POLYPHENOLS AND POLYPHENOLS PLUS ASCORBIC ACID ON OXIDATION AND PHOSPHORYLATION IN THE α-KETOGLUTARATE SYSTEM *

FLASK CONTENT	O UPTAKE	P UPTAKE	P/O
	μ atoms	μ atoms	
Control	15.1	27.0	1.8
" + ascorbic acid (3×10^{-4} M)	10.9	22.6	2.1
" + catechol (3×10^{-4} M)	1.1	2.6	...
" + catechol + ascorbic acid	14.1	31.7	2.2
" + chlorogenic acid (3×10^{-4} M)	2.2	0.0	...
" + chlorogenic and ascorbic acids	15.8	27.6	1.7

* Duration of the experiment 30 min. Reaction mixture of the control same as in table I.

It was still not clear whether the oxidation of the polyphenols was spontaneous or enzymatic. An attempt was made to study this by addition of diethyldithiocarbamate (DIECA), a chelating agent which inhibits copper enzymes (8), to the flasks containing catechol and the α-ketoglutarate-oxidase system. In the presence of DIECA there was no inhibition of the α-ketoglutarate system by catechol (table V). This can be interpreted to mean that the inhibition of α-ketoglutarate oxidation by catechol is dependent on the oxidation of catechol by polyphenolase or copper. There was probably no free copper present, since the mitochondrial preparations were made in the presence of EDTA and all solutions were prepared with doubly glass-distilled water. Therefore, it seems that catechol was oxidized by polyphenolase. However, the possibility of oxidation by a metal-protein-bound moiety exists.

TABLE V

EFFECT OF DIECA ON CATECHOL INHIBITION OF THE α-KETOGLUTARATE SYSTEM

FLASK CONTENT	Q_{O_2} (N)
Control*	564
" + DIECA (6×10^{-5} M)	627
" + catechol (3×10^{-4} M)	0
" + catechol + DIECA	520

* Reaction mixture same as in table I.

DISCUSSION

Attempts have been made by plant biochemists to obtain high efficiencies of phosphorylation similar to those reported for animal tissue (7, 9). With mung bean (4) the P/O ratio was found to be 1, while for the avocado (3) and the cauliflower (10) occasional values exceeding 2 were reported. Recently a P/O of 3.0 was obtained for lupine mitochondria (5). This study with sweet potato indicates that consistently good rates of phosphorus uptake can be achieved with a reconstituted mitochondrial preparation. Although, in the one-step oxidation from α-ketoglutarate to succinate, P/O ratios approaching 3 were obtained, the evidence from DNP inhibition indicates that nearly half of the phosphorylation takes place at the substrate level. This implies that the electron-transport chain is not phosphorylating at maximum capacity.

The data on inhibition of oxidative phosphorylation by polyphenols and its prevention by ascorbic acid can be used as a partial explanation of the course

of senescence in plant tissue. In the normal active cell the oxidation of polyphenols must be coupled to a system that immediately reduces the quinone formed. Ascorbic acid or similar reducing substances may function in this capacity. Darkening due to polymerization of quinones is, therefore, not observed in young normal cells, though the cells may contain polyphenols and polyphenolases. However, if ascorbic acid, or other specific reducing systems, is lost during the aging process, quinones would gradually build up in the cells. The presence of quinones, which rapidly polymerize, results in inhibition of both oxidation and phosphorylation of the mitochondrial system and eventually causes death.

Ezell and Wilcox (6) presented data showing that about 50 % of the ascorbic acid in sweet potatoes was lost in storage. Other data indicate that ascorbic acid declines in plant tissues with age (14). The darkening generally observed when cells are injured and exposed to the atmosphere may be due to the abundance of oxygen which causes the oxidation of the quinone-reducing systems. This consequently allows polymerization of quinones. In general darkening is characteristic of aged tissue. The build-up of quinones during the aging process is probably gradual and does not become detrimental until a concentration of approximately 10^{-4} M is reached. The scheme suggested proposes a possible role of the polyphenol-ascorbic acid systems in senescence which merits further study.

Summary

A mitochondrial system from sweet potatoes, prepared in EDTA-sucrose, carried out oxidative phosphorylation at very good rates during oxidation of α-ketoglutarate. The oxidation and phosphorylation proceeded at constant rates for at least 1 hour. In this time approximately 60 micro-atoms of phosphorus were taken up and a P/O ratio of approximately 2 was obtained. In the presence of malonate the P/O ratios approached 3.

DNP uncoupled oxidation from phosphorylation at concentrations of 1×10^{-4} M, 3×10^{-4} M, and 5×10^{-4} M. Concentrations lower than 1×10^{-4} M had little effect.

The polyphenols, catechol and chlorogenic acid, in concentrations of 3×10^{-4} M almost completely inhibited phosphorylation and oxidation during the dissimilation of α-ketoglutarate by the mitochondrial system. This inhibition was prevented by ascorbic acid or DIECA, suggesting that the quinone form is the inhibitor.

A scheme which attributes senescence in plant cells to interaction of the polyphenol-ascorbic acid systems was proposed.

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