OXIDATION AND PHOSPHORYLATION BY SUBCELL PARTICLES FROM REPRESENTATIVE MEMBERS OF THE PLANT KINGDOM

by

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INTRODUCTION

Particulate fractions from a number of plants were isolated by Bhagvat and Hill (6, pp.112-120). These workers used grinding, filtration, and differential centrifugation for the isolation of their particulate enzyme preparation. Cytochrome c, cytochrome oxidase, and succinic dehyrogenase were found present in the preparations obtained from barley, wheat, corn, a number of types of beans, leeks, onions, dandelions, kale, and rhubarb. This was one of the first reports that certain enzymes and cofactors involved in the Krebs tricarboxylic acid cycle (TCA cycle) could be demonstrated in subcell particles from plants.

Stafford (39, pp.696-737) carried out an extensive study of the fractions that could be obtained by blending (in a Waring blendor), filtration, and differential centrifugation of plant material from etiolated pea seedlings, Pisum sativum. Cytochrome oxidase and succinic dehydrogenase were found by Stafford (39, p.710) in particles isolated by centrifugation between 4,000 and 6,000 x g. These particles appeared to be functionally

similar to liver mitochondria. Electron micrograph pictures (39, p.720) showed the particles to be spheres ranging from 0.1 to 1.0 microns in diameters. Ascorbic acid oxidase, amylase, and phosphorylase were found predominantly in the final supernatant obtained after centrifugation at 60,000 x g (39, pp.712-718).

Millerd, et al (32, pp.855-862) used the sand, pestle, and mortar procedure of Kennedy and Lehninger (24, p.959) with differential centrifugation to obtain enzyme particles from etiolated mung bean seedlings, Phaseolus aureus. These particles could carry out the complete oxidation of pyruvate by way of an apparent TCA cycle. Other particulate preparations with similar activity have been obtained by Davies from pea seedlings (18, pp.173-183), by Millerd, Bonner, and Biale from avocado fruit, Persea americana, (31, pp.521-531) and by Laties from cauliflower buds, Brassica oleracea, (26, pp.557-575). All of these investigators used the procedure of Kennedy and Lehninger (24, p.959) for isolation of the active particles. Brummond and Burris (9, pp.754-759), using the same method, demonstrated that mitochondria isolated from etiolated lupine cotyledons, Lupinus albus, could catalyze the oxidation of the individual acids of the TCA cycle.

Beaudreau (3, pp.32-102) and Beaudreau and Remmert

(4, pp.469-485) have shown that the main reactions of the TCA cycle are catalyzed by a particulate fraction obtained from etiolated seedlings of Black Valentine beans, Phaseolus vulgaris. Beaudreau used a Waring blendor to comminute the plant material. It is interesting to note that Beaudreau's preparation contained a larger number of active enzymes than did that of Stafford (39, p.698) who also used a Waring blendor.

The blendor method of Beaudreau was thought to be superior in certain respects for the isolation of subcell particles from plants which are able to carry out the reactions of the TCA cycle (4, p.484). The work described in the first section of this thesis was an attempt to show that the blendor method developed by Beaudreau could be used with minor modifications to obtain active particulate preparations from species in several classes of the plant kingdom.

METHODS AND MATERIALS

SELECTION AND PREPARATION OF PLANT MATERIAL

Corn, morning glory, and mung bean seeds were treated for ten minutes with a 0.5% solution of NaOCl. They were then washed, planted in moist vermiculite, and grown in the dark in a constant temperature chamber (27°C). The corn and mung bean seedlings were harvested after

ten days. The morning glory seedlings were used when they were between two and three inches in length. After removal of the cotyledons, the remaining aerial portions of the plants were washed and used in each case.

New spring Douglas fir pine needles were removed from healthy trees in the field not more than three hours previous to their use. Most or all of the fibrous stem and petiole tissue was removed. The remaining needles were washed with water and then ground and blended.

Young photosynthesizing poppy plants were harvested and washed, and the leaves and terminal meristems were used directly.

Cabbage, spinach, onion, and lettuce were purchased from a local wholesale market. These plant materials, onion excepted, were harvested not more than two weeks prior to their use. The entire leaves of the plants were used. The cabbage and lettuce preparations were made not only from their respective heads but also exclusively from outer green leaves.

The fern and peppermint plants were grown in the green house in the light. The same group of fern and peppermint plants was used for all of the preparations involving each species. Only newly formed leaves were used in both cases. The fern leaflets were stripped from the stem and used without washing.

HOMOGENIZATION AND CENTRIFUGATION

The homogenizing medium contained 1.0 M sucrose and 0.1 M potassium phosphate at pH 7.0 for all of the different preparations with the exception of those from corn. Sucrose at a concentration of 1.5 M and containing 0.1 M potassium phosphate at pH 7.0 was used for corn.

The amounts of medium, amounts of plant material used, and the periods of homogenization are shown in Table 1. Four equal portions of plant material were used in each experiment. The material was cut into pieces smaller than one centimeter square by using either scissors or a knife and chopping board. Each portion was then blended with the indicated amount of medium in a one liter capacity Waring blendor with a "Cenco-pinto" blade assembly. The blendor was operated at full speed at all times. The time and manner of blending was critical. During homogenization the blendor motor and bowl were rocked as a unit to throw the material into the blades. With a little practice this rocking could be accomplished without danger of stripping the square drive shaft on top of the blendor motor. Homogenization was done in short consecutive time intervals, and after each of these the material in the blendor was stirred and pushed from the walls into the bottom of the blendor jar. After blending,

Table 1

Summary of procedures for homogenizing plant material

Plant	Experiment number from Table 4	Weight of plant material[a]	Volume of homogenizing medium[a]	Homogenizing time[b]
		gm.	ml.	sec.
Ferns	1, 2	80	80	60 (10-10-10-10-10-10)
Ferns	3	50	100	60 (10-10-10-10-10-10)
Douglas fir[c]	4	50	50	35 (5-10-10-10)
Corn	5, 6	100	35	20 (10-5-5)
Onion	7	100	25	10 (5-5)
Spinach	8, 9	80	75	15 (5-5-5)
Poppy	10	75	70	25 (5-10-10)
Cabbage	11, 12, 13, 14	110	55	15 (5-5-5)
Mung bean	15, 16	80	45	6 (1-5)
Morning glory[c]	17	42	80	6 (1-5)
Peppermint	18, 19	60	100	30 (10-10-10)
Lettuce	20, 21, 22	100	25	12 (6-6)

[a] In each experiment four homogenates were prepared using the indicated amounts of plant material and medium for each. The four homogenates were combined by straining them into the same container.

[b] The numbers shown in parentheses following the total time of homogenization indicate the increments of the total time after each of which the material in the blendor was stirred and cleaned from the walls of the blendor jar.

[c] The Douglas fir needles and the morning glory seedlings were passed through a hand meat grinder prior to their introduction into the blendor.

each portion of the material was strained through six layers of 40-grade cheesecloth, and all of the homogenates were combined. The homogenate was then subjected to differential centrifugation as described by Beaudreau (3, p.32). Homogenization was carried out in a walk-in cold room held at 0° to 5° C, all solutions and plant materials being chilled to 5° C before blending. The centrifugation was carried out in a Servall refrigerated centrifuge equipped with a type SS-1 superspeed rotor. The temperature was kept between 0° and 5° C throughout all operations.

SUSPENSION AND ANALYSIS

The enzyme particles obtained in each experiment were washed using volumes of homogenizing medium as follows: 40 ml. for fern, Douglas fir, onion, spinach, cabbage, morning glory, and peppermint preparations; 20 ml. for poppy, mung bean, and lettuce preparations; and 48 ml. for corn preparations. Washing consisted of suspension in the fresh medium followed by resedimentation at 14,000 x g. The enzyme precipitate was then suspended in a volume of homogenizing medium sufficient to permit addition of two milliliters of the suspension to each Warburg flask, plus a sufficient amount for nitrogen determinations. A loose fitting Potter-Elvehjem homogenizer was used to accomplish the suspension. The amount

of nitrogen in the enzyme preparations was determined by the semimicro-Kjeldahl method.

CONDITIONS OF GAS MEASUREMENT

Warburg constant volume respirometers with air as the gas phase were used for all gas exchange measurements. The liquid volume in the main compartment of each flask was 3.2 ml.; 0.2 ml. of 20% KOH was added to each center well along with a small folded square of filter paper. The bath temperature in all experiments was 30° C.

The concentrations of solutes in each flask were as follows: 0.0044 M $MgSO_4$; 0.0016 M Mn SO_4; 0.075 mg. cytochrome c per ml.; 25 parts of terramycin per million; 3.2×10^{-3} M Na_2 ATP · $2H_2O$ (adenosine triphosphate); 0.625 M sucrose; 0.0625 M potassium phosphate buffer, pH 7.0; 2×10^{-4} M DPN (diphosphopyridine nucleotide); 2×10^{-5} M TPN (triphosphopyridine nucleotide); 2.5×10^{-3} M GSH-Na (reduced glutathione, sodium salt); 1.4×10^{-3} M TPP (thiamine pyrophosphate); 0.125 mg. of a Co A (coenzyme A) concentrate per ml. The Co A concentrate was an Armour product obtained from liver and containing 13 Lipmann units of Co A per mg., less than 4% TPN, and less than 7% DPN. The DPN, TPN, TPP, Co A, and ATP solutions were made up fresh for each experiment. All of the other solutions were kept frozen when not in use and were made up fresh every three weeks. The DPN, TPN,

TPP, Co A, GSH-Na, ATP, cytochrome c and terramycin·HCl were obtained in the same purity and from the same companies as reported by Beaudreau (3, pp.6-7). All of the substrates were added as their potassium salts, with the exception of pyruvate which was added as the sodium salt.

PRELIMINARY EXPERIMENTS

In attempting to use the Waring blendor for comminution of a variety of plant materials, it soon became apparent that the amounts of plant material and homogenizing medium, and the time of blending, had to be varied in accordance with the type of tissue under study. Some effort was therefore devoted to developing a systematic method of predicting the best conditions for homogenizing material being studied for the first time. This objective was only partially realized, but a description of the approach may be helpful to others contemplating similar work. It should be mentioned also that the procedures summarized in Table 1 were developed as a result of this preliminary work.

In the first approach to this problem it was assumed that comparable results might be obtained, in going from one species to another, by maintaining a constant percentage of plant dry matter in the homogenizing mixture. To this end, the dry matter contents of the fresh plant materials were determined. Calculations were then made

to determine the amounts of plant material and medium to be used with each species to establish the dry matter concentration present in Beaudreau's homogenates of Black Valentine bean seedlings. This approach was soon abandoned because it became obvious that the dry matter content of the homogenates was only one of several variables affecting the process of comminution.

The method finally adopted for determining the proper amounts of homogenizing medium and plant material was one of running each plant through a certain set of variables. The first variable selected for study was the amount of plant material to be placed in the blendor as one charge. Several trials were conducted using different amounts of plant material and enough medium for good blending in 10 to 20 seconds. No tests for enzymatic activity were made in these preliminary experiments. The most desirable amounts of plant material and medium were judged tentatively as those giving the best blending in a given period of time. At this point the volume of homogenizing medium and time of blending were held constant. A number of particulate fractions were then prepared from different weights of plant material and these were compared for their oxidative activity using Warburg flasks containing the proper medium and substrates. Table 2 shows the type of results obtained.

Table 2

Oxidative activity of particulate fractions prepared from different amounts of plant material

Plant	Weights of tissue	Volume of homogenizing medium	Endogenous O_2 uptake	O_2 uptake with substrate	Net O_2 uptake
	gm.	ml.	µl.[a]	µl.	µl.
Peppermint	30	105	19	39	20
	40	105	30	53	23
	50	105	88	116	28
Ginko	8	90	-	165	-
	12	105	-	69	-
	16	105	-	91	-
	32	105	-	153	-

[a] The following abbreviations are used thoughout this thesis: microliters, µl.; micromoles, µM. or µmoles; microgram atoms, µA. or µatoms.

Next the weight of plant material and volume of homogenizing medium were held constant at the optimal levels determined previously, and the time of homogenization was varied. Again the rate of O_2 uptake in the Warburg flasks was used as the criterion for selecting the proper time. Table 3 shows the results of a few experiments of this type. After going through this crossed variable method twice, a fair estimate of the proper amounts of medium and plant material and of the optimal homogenizing time, could be made. These amounts and times were then used routinely to obtain active fractions for demonstrating substrate oxidation.

It should be mentioned that as experience was gained with the blending of plant material, a reasonably good estimate or prediction of the best conditions could be made from the ease of blending and the appearance of the homogenates. Usually it was found that larger quantities of active particles were obtained when the total volume in the blendor was made up from the maximum amount of plant material and the smallest feasible volume of homogenizing medium that permitted complete blending.

Table 3

Oxidative activity of particulate fractions prepared with different periods of homogenization

Plant	Total time of homogenization	Nitrogen present	Endogenous O_2 uptake	Substrate O_2 uptake	Net O_2 uptake
	sec.	mg.	µl.	µl.	µl.
Peppermint	6	-	54	62	8
	15	-	88	91	4
	25	-	103	120	17
	35	-	121	125	4
Ferns	5	4.24	173	228	55
	10	3.30	105	248	143
	30	-	136	157	21
	45	5.02	228	220	0
Corn	4	-	75	138	63
	10	-	80	125	45
	18	-	143	193	50
	26	-	126	218	92

RESULTS AND DISCUSSION

PARTICULATE OXIDATIVE ACTIVITY OBTAINED FROM REPRESENTATIVE PLANTS

This work was conducted in the form of a survey in which readily available plants were selected from Filicineae, Gymnospermae, and Angiospermae classes. Table 4 shows some of the results obtained from the plants selected for study. The data are arranged in the ascending order in which the plants are classified in the plant kingdom. Each value for O_2 uptake represents the average of results from at least two flasks unless indicated otherwise. More than two active enzyme preparations (in many cases more than five) showing substrate effects were obtained from each of the species with the exception of onions and California poppies.

A number of preparations were made from calla lillies and morning glory leaves. The preparations from calla lillies were inactive, and those from morning glory leaves had only slight activity. It is worth mentioning that the homogenates from these two species were highly viscous and that the high viscosity was probably caused by the presence of large amounts of polysaccharide-like materials. In any event it was observed that the chloroplast fraction sedimented at much higher speeds, in these viscous homogenates, than was

Table 4

Oxygen uptake catalyzed by subcell particles from plants

Experiment number	Plant species,[a] length of experiment, nitrogen[b]	Substrate used and amount added in μmoles	μl. O_2 uptake above endogenous	% Oxidation[c]
	Filicineae			
1.	Fern *Pteris tremula* 10 hrs. 2.0 mg. N	Pyruvate 20	268	24
		Malate 10	351	52
		Malate 10, pyruvate 20	977	58
		α-Ketoglutarate 10	592	66
		Endogenous	246	-
2.	Fern *Pteris tremula* 6½ hrs.	Succinate 10	403[d]	51[d]
		Citrate 40	1025[d]	26[d]
		Malate 40	580	21
		Endogenous	227	-
3.	Duckweed fern *Azolla filiculoides* 2 hrs. 3.67 mg. N	Citrate 30	72	2
		Succinate 30	95	4
		Malate 30	77	4
		α-Ketoglutarate 30	39	1
		Endogenous	135	-

Table 4 (continued)

Experiment number	Plant species,[a] length of experiment, nitrogen[b]	Substrate used and amount added in μmoles	μl. O_2 uptake above endogenous	% Oxidation[c]
	Gymnospermae			
4.	Douglas Fir *Pseudotsuga menziesii* 5 hrs.	Citrate 10	43[d]	4[d]
		Endogenous	333	-
	Angiospermae			
5.	Corn *Zea mays* Etiolated seedlings 7½ hrs. 1.60 mg. N	Citrate 20	513[d]	26[d]
		Citrate 30	637[d]	21[d]
		Succinate 30	436	19
		α-Ketoglutarate 30	298	11
		Malate 30	128	6
		Endogenous	264	-
6.	Corn *Zea mays* Etiolated seedlings 8½ hrs.	Pyruvate 20	32	3
		Pyruvate 20, malate 5	185	13
		Malate 5	64	19
		Endogenous	207	-

Table 4 (continued)

Experiment number	Plant species,[a] length of experiment, nitrogen[b]	Substrate used and amount added in μmoles	μl. O_2 uptake above endogenous	% Oxidation[c]
7.	Onion[e]			
	Allium	Malate 10	52[d]	8[d]
	White bulbs	Succinate 10	141[d]	18[d]
	9½ hrs.	α-Ketoglutarate 10	47	5
	0.82 mg. N	Citrate 10	79[d]	8[d]
		Endogenous	88	-
8.	Spinach			
	Spinacia oleracea	Succinate 10	467	60
	7 hrs.	Citrate 10	606	61
	1.75 mg. N	Succinate 10, pyruvate 20	599	31
		Pyruvate 20	52	9
		Endogenous	170	-
9.	Spinach			
	Spinacia oleracea	Citrate 10	215	21
	4 hrs.	Succinate 10	218	28
	2.46 mg. N	α-Ketoglutarate 10	197	22
		Malate 10	126	19
		Endogenous	211	-
10.	California poppy[e]			
	Eschscholzia californica	Citrate 10	26[d]	3[d]
	2 hrs.	Succinate 10	52	7
	1.70 mg. N	α-Ketoglutarate 10	16	2
		Malate 10	7[d]	1[d]
		Endogenous	51	-

Table 4 (continued)

Experiment number	Plant species,[a] length of experiment, nitrogen[b]	Substrate used and amount added in μmoles	μl. O_2 uptake above endogenous	% Oxidation[c]
11.	Cabbage *Brassica oleracea* 6½ hrs. 0.50 mg. N	Citrate 10	1007	100
		Endogenous	128	-
12.	Cabbage *Brassica oleracea* 8½ hrs. 0.96 mg. N	Malate 10	665	99
		Malate 10, pyruvate 20	963	54
		Pyruvate 20	32	3
		Endogenous	157	-
13.	Cabbage *Brassica oleracea* 5½ hrs.	Succinate 10	776	99
		Endogenous	109	-
14.	Cabbage *Brassica oleracea* 5¼ hrs.	α-Ketoglutarate 10	748	83
		Endogenous	142	-
15.	Mung bean *Phaseolus aureus* Etiolated seedlings 6 hrs. 0.59 mg. N	Citrate 10	220	22
		Succinate 5	96	24
		Succinate 5, pyruvate 20	171	11
		Pyruvate 20	32[d]	3[d]
		Endogenous	142	-

Table 4 (continued)

Experiment number	Plant species,[a] length of experiment, nitrogen[b]	Substrate used and amount added in μmoles	μl. O_2 uptake above endogenous	% Oxidation[c]
16.	Mung bean			
	Phaseolus aureus	Succinate 10	441	56
	Etiolated seedlings	Citrate 10	216	22
	6 hrs.	Malate 10	396	59
	0.95 mg. N	α-Ketoglutarate 10	437	48
		Endogenous	217	-
17.	Morning glory			
	Convolvulus	Citrate 10	189	19
	7 hrs.	α-Ketoglutarate 10	56	6
	3.4 mg. N	Endogenous	439	-
18.	Peppermint			
	Mentha piperita	Citrate 10	31	3
	2 hrs.	Succinate 10	12	2
	1.95 mg. N	Endogenous	93	-
19.	Peppermint			
	Mentha piperita	Succinate 10	145	18
	7 hrs.	Citrate 10	10	1
	2.77 mg. N	α-Ketoglutarate 10	137	15
		Endogenous	425	-
20.	Lettuce			
	Lactuca sativa	Malate 10	258	38
	6½ hrs.	α-Ketoglutarate 10	269	30
	0.64 mg. N	Endogenous	60	-

Table 4 (continued)

Experiment number	Plant species,[a] length of experiment, nitrogen[b]	Substrate used and amount added in μmoles	μl. O_2 uptake above endogenous	% Oxidation[c]
21.	Lettuce *Lactuca sativa* 8 hrs.	Citrate 10	575	57
		Endogenous	88	-
22.	Lettuce *Lactuca sativa* 7½ hrs.	Succinate 10	536	68
		Pyruvate 20	68	6
		Succinate 10, pyruvate 20	973	51
		Endogenous	116	-

[a] All preparations were made from actively photosynthesizing stem and leaf tissue unless indicated otherwise.
[b] Nitrogen values indicate the mg. of nitrogen contained in the particulate preparation added to each Warburg flask within a given experiment.
[c] The percentage oxidation was calculated by dividing the microliters (μl.) of O_2 theoretically required for complete oxidation of the substrate into the measured substrate-induced O_2 uptake.
[d] One flask only.
[e] One experiment only.

the case in suspensions of lower viscosity. Therefore it is highly possible that the low activity observed with preparations from calla lillies, and from morning glory leaves, was caused by failure of the mitochondria to sediment from the highly viscous homogenates obtained.

It can be seen in Table 4 that the particulate preparations from fern, corn, spinach, cabbage, mung bean, and lettuce caused a large increase in O_2 uptake when any one of a number of the TCA cycle intermediates was added. With each of these six plant particulate preparations the O_2 uptake obtained from the combination of pyruvate with a four carbon acid was greater than the sum of the O_2 uptakes obtained for pyruvate alone and the four carbon acid alone. This gave a good indication that the condensing enzyme of the TCA cycle was operating. The large amounts of oxygen uptake, induced by the intermediates involved in the main oxidative steps of the cycle, strongly suggest that the entire cycle was operating in these six plant preparations.

Catalysis of endogenous O_2 uptake by TCA cycle members could account for a fraction of the substrate effects observed. The fact that a number of the cycle intermediates induced O_2 uptake, however, argues for at least a partial oxidation of each member of the cycle. Experiments which will be mentioned later in this

thesis tend to substantiate the conclusion that not more (and possibly much less) than 28% of the substrate induced O_2 uptake arose from a catalyzed endogenous respiration.

Attention should be pointed to the fact that rather long incubation periods were used in all of the O_2 uptake experiments reported in Table 4. The stability of the subcell particles was surprisingly good. The long incubation periods were required to obtain the high percentage oxidation of substrates, desired in these experiments. Higher rates can be obtained and shorter incubation times can be permitted by using larger amounts of plant particulate material in the Warburg flasks. This was not practiced, however, because of the limited centrifuge capacity and the number of substrates and other variables that needed to be compared within a given experiment.

Beaudreau (3, pp.81-83) reported that long incubation periods, similar to those conducted in this work, resulted in a pronounced bacterial growth and an increasing rate of "endogenous" O_2 uptake. Beaudreau recommended the use of terramycin to overcome this bacterial O_2 uptake. In all of the experiments conducted by the author, terramycin was used and the rates of O_2 uptake were always plotted to see if a later increase in rate took place. In no instance did bacterial contamination

appear to contribute to the effects measured.

The O_2 uptake activity obtained with particles from Douglas fir, onion, poppy, morning glory, and peppermint, though not convincing, is at least suggestive evidence for the operation of the TCA cycle in these plant preparations.

The very high rates obtained with cabbage particulate preparations, their high stability plus the ease with which this plant material can be obtained all the year round, instigated further, more extensive work on the reactions catalyzed by the cabbage particles. This additional work is included in the latter sections of this thesis.

Another fact worthy of emphasis is that most of the particulate preparations were obtained from green photosynthesizing tissue. That particles catalyzing the TCA cycle can be obtained from green leaves is contrary to what was observed by Brummond and Burris (9, p.757) with lupine particulate preparations. These workers found that only α-ketoglutarate was oxidized by particulate preparations from green lupine leaves, and even this reaction occurred at a slow rate. No activity was obtained from TCA members with particles from photosynthesizing lupine cotyledons. It was found by the author that young cabbage seedlings, when grown in the light,

contained a rather large amount of fibrous material which interfered considerably with the homogenization. The fraction obtained after the high speed centrifugation was not homogeneous. Small irregular fragments of cellulose-like material were observed to be present when this fraction was viewed in a light microscope using oil immersion. The usual cabbage preparations appeared to be homogeneous suspensions of very fine particles that were just visible. Active particulate preparations could be obtained from young photosynthesizing cabbage seedlings when only succulent nonfibrous tissue was used; preparations from highly fibrous tissues generally had little or no activity.

RATE COMPARISONS

Table 5 shows some of the rates found by the author with various plant preparations during the first thirty minutes of incubation. It is very probable that these rates are not the maximum ones obtainable with the particulate preparations studied since low concentrations of substrates were used.

The tissue slice preparations mentioned in Table 5 were obtained by two different methods. On the one hand, slices were prepared using the Stadie-Riggs microtome; on the other, they were prepared by hand using a razor

Table 5

Rates of oxygen uptake for various plant preparations

Experiment number[a]	Type of preparation	Substrate used and amount added in μmoles	O_2 uptake above endogenous[b] μl/hr/mg N
1.	Fern particles	Pyruvate 20	31
		Malate 10	16
		Malate 10, pyruvate 20	128
		α-Ketoglutarate	17
6.	Corn particles	Citrate 30	184
		Succinate 30	224
		Malate 30	49
		α-Ketoglutarate 30	54
12.	Cabbage particles	Citrate 10	836
24.	Cabbage particles[c]	Citrate 10	1020
25.	Cabbage leaf slices[d]	Malate 10	108[e]
	Cabbage leaf slices[f]	Malate 10	340[e]
17.	Mung bean particles	Succinate 10	200
		Malate 10	116
		α-Ketoglutarate 10	158

Table 5 (continued)

Experiment number[a]	Type of preparation	Substrate used and amount added in μmoles	O_2 uptake above endogenous[b] μl/hr/mg N
21.	Lettuce particles	Malate 10	39
		α-Ketoglutarate 10	95

[a] All data listed under a given experiment number in this table and in Table 4 were obtained in a single experiment.
[b] Each value represents the average of results from two Warburg flasks.
[c] The standard method for obtaining the subcell particles was modified in that the cabbage was passed through a meat grinder prior to blending in the usual manner.
[d] Prepared with a Stadie-Riggs slicer.
[e] These values include the endogenous O_2 uptake.
[f] Prepared using a razor blade.

blade. In the latter case the slice thickness varied between 0.25 and 0.50 mm. All of the slices were prepared from leaf tissue devoid of large veins and the slices were made in the plane of the leaf blade. The same number of slices was introduced into each of the Warburg vessels, which contained the usual cofactors and substrates. The flasks containing the slices were incubated at 30° C in a Warburg bath. At the end of the incubation the slices were removed and washed, and their nitrogen content was determined. An extremely small substrate induced oxygen uptake occurred with these tissue slice preparations, and most of their oxygen uptake was of an endogenous origin.

It is evident from Table 5 that the highest $Q_{O_2}(N)$ values (µl. O_2/hr./mg. N) found by the author were obtained with subcell particles when the meat grinder was used prior to blending. These values, to the author's knowledge, are the highest ever reported for any plant particulate preparation. In the experiment reported (Experiment 24, Table 5) 759 µl. of oxygen, in addition to an endogenous value of 77 µl., were taken up in $6\frac{1}{2}$ hours by the meat grinder particulate preparation. Another preparation obtained by the usual method took up 1007 µl of oxygen in $6\frac{1}{2}$ hours, in addition to an endogenous of 128 µl. The big difference between the meat grinder preparation and the usual preparation was in the

nitrogen values found. Only 0.2 mg. of nitrogen was found to be present in 2 ml. of the enzyme suspension from the meat grinder preparation and 0.5 mg. of nitrogen was found in 2 ml. of the usual preparation. These results indicate that a higher rate of O_2 uptake per unit of nitrogen was obtained after use of the meat grinder. It is apparent, however, that a lower yield of active particles was obtained when the meat grinder was used, which explains the lower net oxygen uptake data at 6½ hours. The reason the meat grinder was not employed routinely was because of the limited capacity of the centrifuge. Larger quantities of active particulate material were sought rather than maximum $Q_{O_2}(N)$ values.

The other $Q_{O_2}(N)$ values given in Table 5 for subcell particles, though low when compared with the more active preparations, are of the same order of magnitude as some values reported in the literature for animal preparations (35, p.143, p.147, and p.148; 20, p.625; 38, p.38).

When one compares the $Q_{O_2}(N)$ values found for cabbage leaf slices with those found for cabbage particulate preparations (Table 5), at least one thing stands out. The rate per unit of nitrogen for the separated particles was greater, even though the particles were

probably surrounded by less substrate than when whey are present in intact cells. From this, one could say that a concentration of active enzyme particles could have taken place. This is not unreasonable since in the whole cell nitrogen compounds are present which are not involved with the operation of the TCA cycle. The actual validity of this contention and the extent of concentration of active particles is difficult to determine. In the whole cell mechanisms for the control of the TCA cycle are presumably at work, whereas in the isolated particles no such control may be present. If this is the case then the isolated particle could respire faster because of the absence of the normal cell control. Cofactor permeability of the cell wall, differences in dispersion of the two types of preparations and the resulting different rates of oxygen diffusion, and the unnatural conditions to which the particles are subjected are only a few of the other factors that could be mentioned to explain this difference between the particulate and tissue slice rates of O_2 uptake.

A few $Q_{O_2}(N)$ values reported by other workers are recorded in Table 6 together with the highest rate obtained with cabbage particles. Calculations were necessary to put these values on the same basis for comparison. It is apparent that the rates of oxygen

Table 6

$Q_{O_2}(N)$ values for various particulate preparations

Type preparation	Substrate and its concentration molarity	Temperature deg. C	$Q_{O_2}(N)$	Reference
Guinea pig heart muscle mitochondria	Succinate .0067	30	550	(35, p.143)
Rat sarcosomes	α-Ketoglutarate .007	25	312	(38, p.34)
Rat liver particles	Succinate .050	38	2020	(20, p.625)
Mung bean hypocotyl particles	Succinate (not given)	30	773	(30, p.161)
Black Valentine particles	Succinate .0150	30	565	(3, p.87)
	Succinate .0094	30	364	
	Citrate .0094	30	338	
Cabbage particulate preparations	Citrate .0031	30	1020	(Table 5)

uptake obtained in this study are comparable to those reported by other workers for both animals and plants. It is interesting to note that the oxygen uptake rates occurring generally in plant particulate preparations are as large as those reported for animal particulate preparations. The high value reported in Table 6 for rat liver particles can undoubtedly be explained by the higher temperature and the higher substrate concentration used.

The highest $Q_{O_2}(N)$ value for which data was given by Millerd (30, p.157) for particulate preparations from mung beans was 262. Millerd used 0.02 M succinate to obtain this value. Millerd did not state the substrate concentration used for obtaining the $Q_{O_2}(N)$ value of 773 shown in Table 6. The $Q_{O_2}(N)$ values obtained by the author for mung bean particles were between 100 and 200 with substrate concentrations of 0.0031 M (Table 5). When considering only the rate of oxygen uptake one can conclude that mung bean particulate preparations obtained by using the blendor method are comparable to those obtained by Millerd using the sand, pestle, and mortar method.

LIGHT EFFECT ON A SPINACH PARTICULATE PREPARATION

Four 300-watt flood lights were directed from above on a circular Warburg bath having a radius of 12 inches. Six 15-watt bulbs were fitted at equal intervals around the post in the middle of the Warburg bath. Half of the Warburg flasks used in the experiment, and the exposed parts of their manometers, were painted with three coats of black asphaltum.

The spinach particulate fraction was prepared in the usual manner and aliquots were introduced into the Warburg flasks with the normal cofactors. The "direct method" of Dixon and Warburg (42, pp.17-19) was then used for manometric measurements of CO_2 evolution and O_2 uptake. During the experiment all of the lights were kept operating continuously. Acid was tipped into the reaction mixture of certain flasks, in some cases at the beginning and in others at the end of the experiment, to enable a correction to be made for the retained CO_2 as described by Umbreit, et al (42, p.19). The painted and the unpainted flasks were treated in the same manner. The only difference between the two sets of flasks was the light transmitted by those that were unpainted.

Using the data obtained, the values for the substrate induced O_2 uptake in the light and in the dark were

calculated by subtracting the endogenous values. The net values for the O_2 uptake induced by citrate and by succinate are graphed in Figure 1. It is apparent that the light caused a 69% reduction in the citrate induced O_2 uptake and a 60% reduction in the succinate induced uptake.

Calvin and Massini (13, pp.445-457) demonstrated that when $C^{14}O_2$ was fixed by algae exposed to strong light, the TCA cycle intermediates became labeled only very slowly. When the light was turned off, the $C^{14}O_2$ readily entered the TCA cycle intermediates. These workers suggested a light inhibition of the decarboxylation of pyruvic acid, caused by a light-catalyzed reduction of thioctic acid. It therefore appears reasonable to suggest that the light inhibition of substrate oxidation, shown in Figure 1, could have been caused by the reduction of thioctic acid and the consequent limitation of pyruvate decarboxylation.

NATURE OF THE ENDOGENOUS METABOLISM

It will be recalled that an endogenous O_2 uptake was found with every plant particulate preparation reported in Table 4. At least three possible explanations of this endogenous oxidation appear worthy of consideration, namely: that the particles had not been washed

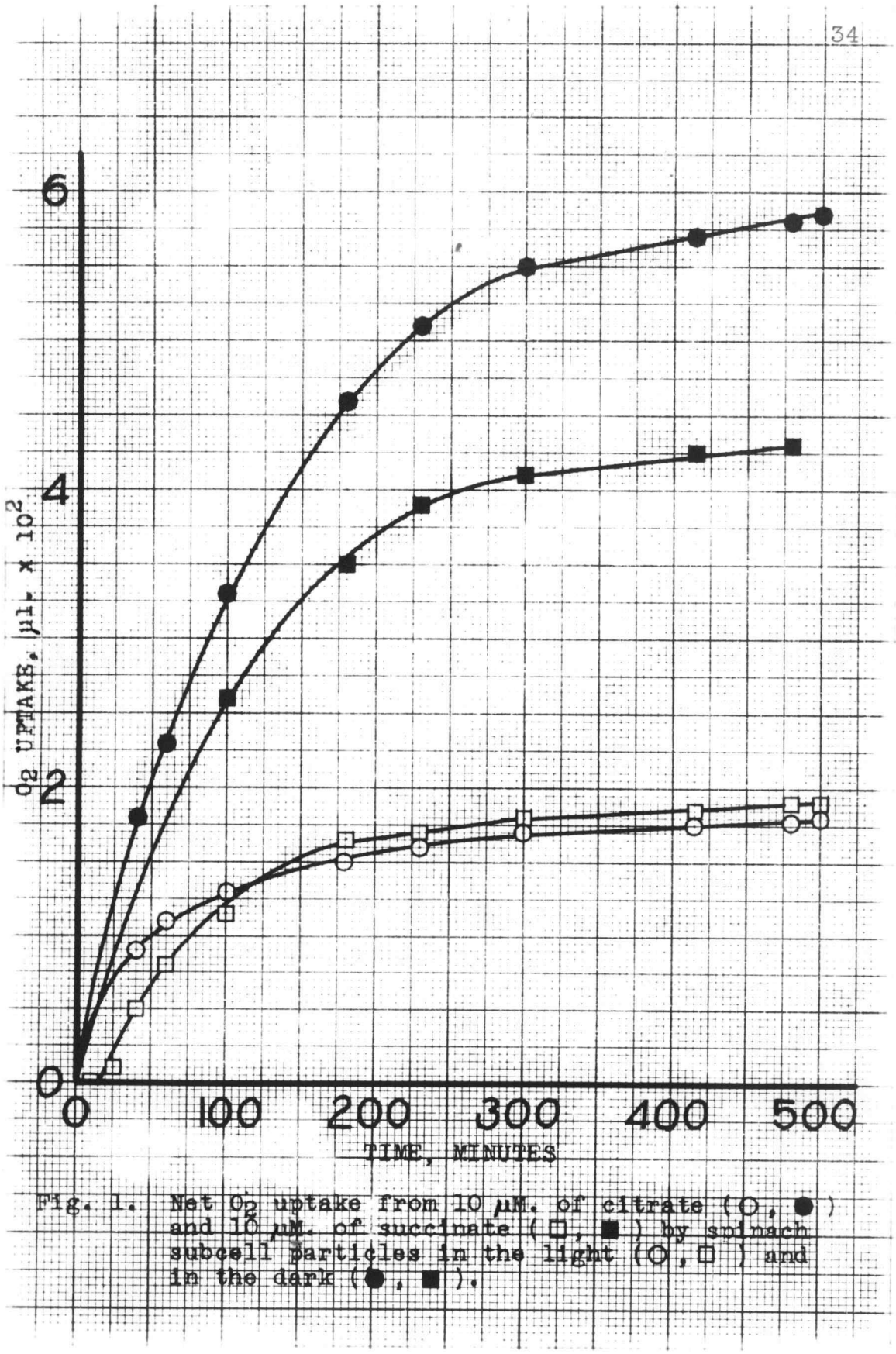


Fig. 1. Net O_2 uptake from 10 µM. of citrate (○, ●) and 16 µM. of succinate (□, ■) by spinach subcell particles in the light (○, □) and in the dark (●, ■).

completely free of endogenous TCA cycle intermediates, that the particles catalyze oxidative reactions on material other than TCA cycle intermediates, and that other oxidative enzymes not associated with the particles were present in the preparations. The exact nature of the endogenous metabolism was not determined in this study. However, certain indications were obtained, and these will be recorded for their possible value to future workers.

Very interesting data concerning the endogenous metabolism were obtained in the experiment discussed in the previous section. Figure 2 shows the endogenous O_2 and the total CO_2 exchange data from the experiment, partially recorded in Figure 1, in which the spinach preparation was used. No endogenous CO_2 flasks were prepared in that experiment; therefore the CO_2 data given in Figure 2 represent the total amounts evolved. It can be seen that the endogenous O_2 uptake rate in the light increased towards the end of the experiment. In a very similar manner the rate of total CO_2 evolution in the light increased after 230 minutes and until the end of the experiment. The rates of CO_2 evolution and endogenous O_2 uptake in the dark followed a form similar to that obtained for substrate induced oxygen uptake as shown in Figure 1. From this data one can conclude that the increased rate of O_2 and CO_2 exchange after 230 minutes

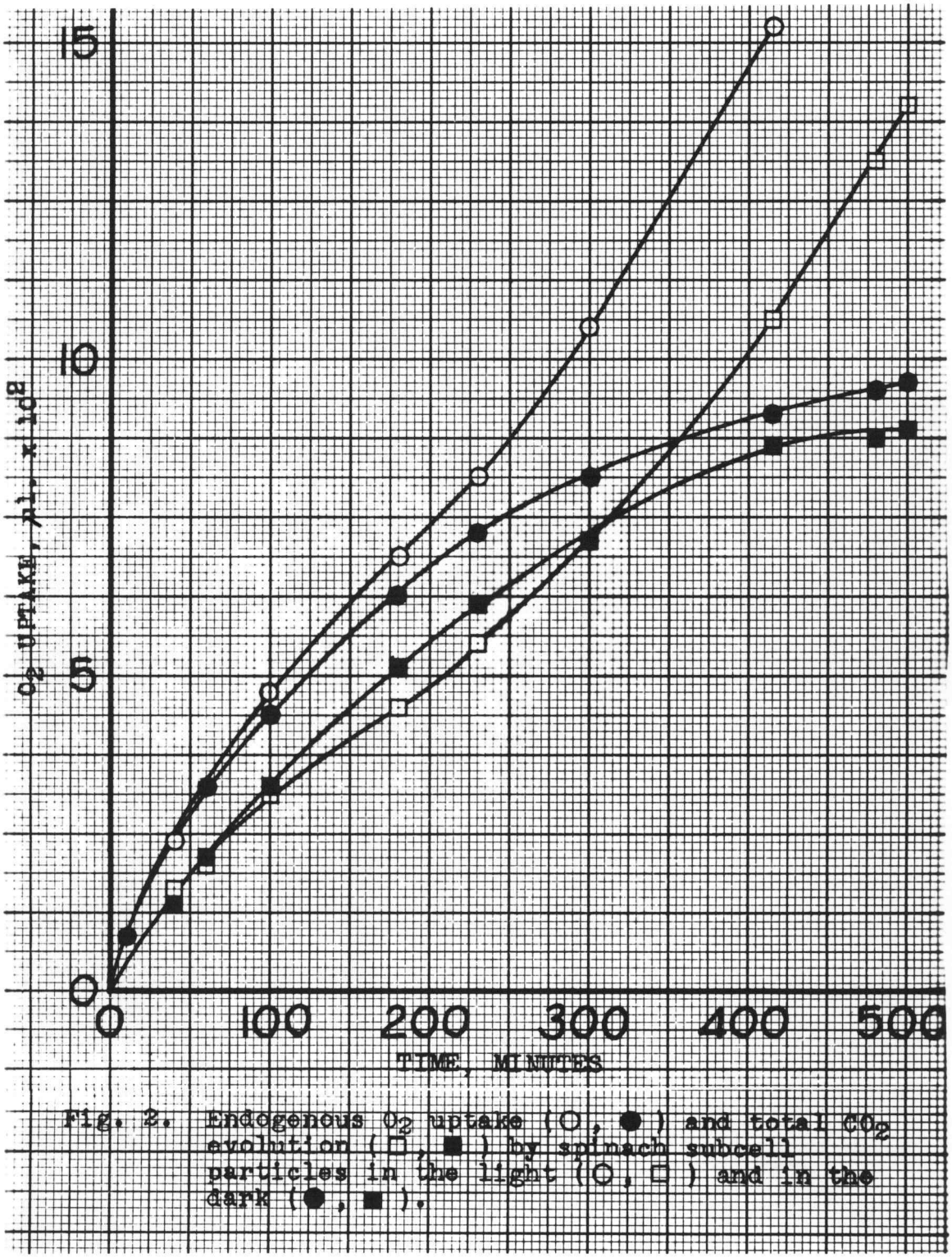


Fig. 2. Endogenous O_2 uptake (○, ●) and total CO_2 evolution (□, ■) by spinach subcell particles in the light (○, □) and in the dark (●, ■).

of incubation was caused by a light stimulation of the endogenous respiration. Similar photocatalysis of endogenous O_2 uptake was observed with both lettuce and fern preparations; however, the effects were not as pronounced as those reported for spinach.

Since no increase in oxidation of TCA members occurred in the presence of light (see Figure 1) it appears that the endogenous respiration was at least partly composed of the oxidation of substrates other than TCA cycle intermediates.

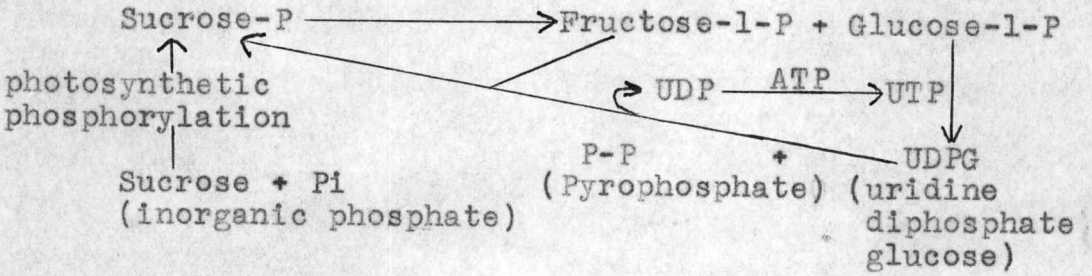
That the endogenous O_2 uptake was not caused entirely by contamination with TCA cycle intermediates is further indicated by the results of experiments in which attempts were made to reduce the endogenous metabolism by more thorough washing of the particles. In one experiment particles from lettuce were washed with four times the usual volume of homogenizing medium. This treatment resulted in only a 7% reduction of the endogenous metabolism obtained after the usual washing procedure. In another experiment, when cabbage particles were subjected to four separate washings, the endogenous respiration was apparently reduced by approximately 20% as compared to that obtained with particles washed once. Such results would appear to indicate that the endogenous O_2 uptake could not arise solely from TCA cycle intermediates since these should diffuse readily into the washing medium.

It is reasonable to speculate that the endogenous O_2 uptake might arise from the metabolism of hexoses or sucrose. With reference to the hexoses, it was observed that when photosynthesizing tissues were used a large amount of starch invariably sedimented during the low speed centrifugation. While this low speed fraction was discarded, the possibility exists that small starch granules remained in the supernatant fluid to be sedimented with the active particles. If such were the case, the starch might be slowly converted to hexoses or hexose phosphates, and these might be metabolized by way of certain pentose conversions (21, pp.661-682) or the pentose cycle. With reference to the sucrose, it is to be recalled that the particles were prepared and incubated with large amounts of sucrose constantly present in the medium. Therefore any enzyme or system capable of causing sucrose oxidation would have ample substrate and could contribute to the endogenous O_2 uptake observed.

Enzymes have been isolated from spinach leaves which can carry out the oxidation of glucose-6-phosphate by way of pentose phosphates (21, pp.661-682). For sucrose to be oxidized through the pentose cycle, a preliminary hydrolysis and phosphorylation would presumably be necessary (37, pp.885-902). Since the spinach preparations contained chloroplasts, the photosynthetic phosphorylation found by Arnon, Daniel, and Whatley

(1, pp.394-396) in whole spinach chloroplasts might be one means by which sucrose could be converted to an intermediate suitable for entering the pentose cycle. If sucrose, glucose, or fructose were linked with this photosynthetic phosphorylation, by being either a primary acceptor or something close to it, it appears probable that some kind of oxidative reaction would result.

Buchanan, et al (12, pp.935-945) identified uridine diphosphate glucose and uridine diphosphate galactose in plants. The hexoses of these compounds became labeled rapidly during photosynthesis in $C^{14}O_2$ (5, p.1714). Because the uridine compounds appeared to precede the labeled sucrose, Buchanan (11, pp.140-149) suggested that they serve as intermediates in sucrose synthesis. In the following scheme all of the reactions, with the exception of that involving photosynthetic phosphorylation, were postulated by Buchanan (11, pp.144-149). These reactions might serve to give one explanation for the suggested photocatalyzed oxidation of sucrose, since the conversion of sucrose to glucose-1-phosphate would permit entry into the pentose cycle.



SUMMARY

The data presented indicate that the reactions of the Krebs tricarboxylic acid cycle are catalyzed by subcell particles which may be obtained from fern (*Pteris tremula*), cabbage, lettuce, corn, spinach and mung bean tissues. Definite but less conclusive evidence is given for catalysis of these reactions by particles from Douglas fir, onion, poppy, morning glory, fern (*Azolla filiculoides*), and peppermint tissues. The three highest classes of the plant kingdom are represented by the twelve species mentioned.

Active particles were obtained from the young, photosynthesizing tissues of certain species, namely, fern, spinach, lettuce, cabbage, Douglas fir, poppy, morning glory and peppermint.

High percentages of added substrates were apparently oxidized in the presence of some of the preparations. The rates of oxygen uptake obtained with particles from cabbage tissues are similar to those reported for subcell particles from animal tissues, when the results are compared on a nitrogen basis.

PART II OXIDATIVE PHOSPHORYLATION BY CABBAGE PARTICULATE PREPARATIONS

INTRODUCTION

Millerd, et al (32, pp.860-861) demonstrated that mung bean particulate preparations could carry out oxidative phosphorylation when TCA cycle intermediates were added. Using P^{32}, these workers showed that radioactivity was incorporated into ATP when α-ketoglutarate was oxidized. ATPase enzyme activity was found to be associated with the particles. The ATPase activity could be inhibited 90% or more by 0.01 M fluoride ion. They found that hexokinase present in the preparations was able to catalyze the formation of hexose-6-phosphate from ATP and hexose. Invertase was also thought to be bound to the particles, but no phosphorylase activity could be demonstrated.

Bonner and Millerd (8, pp.135-148) measured the rate at which mitochondria from mung beans fixed inorganic phosphate (Pi). This fixation occurred when a number of TCA cycle members were oxidized. One mole of Pi was incorporated into an organic form for each atom of oxygen consumed; therefore the highest P:O ratio obtained by these workers was 1. The reaction mixture used by Bonner and Millerd (8, p.144), for demonstrating

oxidative phosphorylation from TCA cycle intermediates, contained 0.2 M sucrose, 0.1 M dextrose, 10^{-3} M AMP (adenosine monophosphate), 10^{-3} M $MgSO_4$, 0.01 M NaF, 0.1 M phosphate buffer, and the enzyme preparation.

Millerd, Bonner, and Biale (31, pp.521-531) used essentially the same method and reaction medium as were used by Bonner and Millerd, for obtaining oxidative phosphorylation catalyzed by mitochondria from avocado fruit. Again the maximum P:O ratios were near 1.

Laties (26, pp.557-575) prepared mitochondria from heads of cauliflower, Brassica oleracea. P:O ratios as high as 2.4 were obtained for α-ketoglutarate oxidation. The reaction mixture contained 2 x 10^{-2} M phosphate buffer pH 7.3, 2 x 10^{-5} M cytochrome c, 10^{-3} M ATP, 1.8 x 10^{-3} M $MgSO_4$, 3.3 x 10^{-4} M DPN (for malate oxidation experiments only), 0.5 M sucrose, 10^{-2} M glucose, 10^{-2} M NaF, and a yeast hexokinase preparation.

Conn and Young (16, pp.195-196) were able to obtain oxidative phosphorylation activity from lupine mitochondria. The reaction medium used contained 0.5 M sucrose, a buffer (either trishydroxymethyl aminomethane or phosphate), $MgSO_4$, AMP, NaF, glucose, and the mitochondria. These workers have not reported the actual concentrations of the substances in the reaction flasks. No improvement in phosphorylation was obtained when either cytochrome c

or Co A was added. These workers stated (16, p.196) "P:O ratios greater than 3.0 are regularly obtained with α-ketoglutarate, and values approaching 2.0 are observed with succinate."

The work reported in this part of the thesis was done in an effort to demonstrate the optimal conditions for oxidative phosphorylation with cabbage particulate preparations and to gain some idea of the magnitude of the P:O ratios that can be obtained when various substrates are present in the reaction medium.

METHODS AND MATERIALS

PREPARATION, SUSPENSION, AND ANALYSIS

Heads of fresh cabbage were obtained and treated in the manner described under "METHODS AND MATERIALS" in Part I of this thesis. A few changes were made in the method for isolating the cabbage particles. Versene, at a concentration of 0.01 M, was used in the homogenizing medium with the usual solutes, and 0.6 M sucrose was used instead of 1 M sucrose in the wash and final suspension mediums. All of the mediums still contained 0.1 M phosphate buffer. After blending and filtration the pH of the homogenate was found to be usually near 5. At this point the pH was adjusted to a value above 6.5 and below 7.0, using K_2CO_3. The strained homogenate was

centrifuged at 3,000 x g for 10 minutes to remove cell debris. The supernatant was then centrifuged at 12,000 x g for 15 minutes to sediment the active particles.

It was found that the loose fitting Potter-Elvehjem homogenizer (P.E.) did not give an adequate, uniform suspension of the enzyme particles, after they had been sedimented and washed. Activity could easily be destroyed or greatly reduced by improper or lengthy homogenization and globules of enzyme particles could be observed even after prolonged homogenization in the P.E. homogenizer.

A new method was developed for the suspending of gummy precipitates. In this method a 20 ml. Gooch crucible was placed in a Walter crucible holder and the whole assembly was fitted into a 125 ml. filtering flask which contained chopped ice and a short 30 ml. graduated cylinder. A mat of fine pyrex glass wool was placed in the bottom of the Gooch crucible. A stirring rod flattened on the end was used to press the glass wool mat into place. The crucible, mat, and graduated cylinder were washed with suspension medium. The precipitate was transferred to the Gooch crucible with the aid of a small amount of the suspension medium. A small additional amount of suspension medium was added, and the mixture was gently stirred, using the flattened stirring rod, without

disrupting the glass wool mat. A vacuum was then applied to force the solution into the graduated cylinder. The glass wool mat was washed twice with homogenizing medium. Each time the stirring rod was used to loosen the particles on the mat. For final dispersion, all of the solution in the graduated cylinder was again passed through the glass wool mat. After this, the mat usually had only a pale green color, and no large globules of particulate material could be seen on the glass wool.

The new method for suspending the particles had a number of advantages. A uniform suspension could be obtained with a minimum of abrasive action. The precipitate and solution were filtered at the same time they were being suspended. The filtration helped to remove any large tissue fragments or cheese cloth threads that accidently might be carried on into the final suspension. The suspension of the precipitate was rapid and efficient. The apparatus was simple and very easy to manipulate.

The total volume in each Warburg flask included 3.2 ml. in the main compartment and 0.2 ml. of 20% KOH in the center well. The solutes in the reaction flasks were (unless indicated otherwise) 0.004 M $MgSO_4$, 0.0016 M $MnSO_4$, 25 parts terramycin per million, 3.2×10^{-3} M K_2 ATP $\cdot$ 2 H_2O, 0.375 M sucrose, 0.0625 M phosphate buffer, pH 6.9, 2×10^{-4} M DPN, 2×10^{-5} M TPN, 2.5×10^{-3}

M GSH-Na, 0.075 mg. cytochrome c per ml., 0.02 M NaF, 0.10 M glucose, and 0.156 mg. of a hexokinase preparation per ml. (Pabst product obtained from yeast and containing 28,000 K.M. units per gram at 30° C). These compounds were obtained from the same sources as those described previously in Part I.

ALIQUOTING METHODS FOR THE PHOSPHORUS DETERMINATION

A number of methods for taking aliquots for the Pi determination were tried. Two methods were finally used.

In the first method the enzyme particulate preparation was added to the flasks containing their reaction mixtures and necessary substrates. The Warburg flasks were fitted to their manometers and allowed to equilibrate with the bath temperature for six minutes. At the end of this time the flasks were removed one at a time and a 0.5 ml. aliquot was taken from each for the zero time Pi determination. Each flask was then fastened to its manometer and returned to the Warburg bath. After this the usual six minute equilibration period was allowed, the manometers were closed, and the manometer readings were recorded at appointed time intervals. At the end of the desired incubation time, a single manometer was read and opened, the flask was again removed, and again a 0.5 ml. aliquot was withdrawn. This final aliquoting process was repeated for each manometer and

flask. The timing between aliquoting and reading of the manometers was maintained as uniformly as possible. Corrections for the O_2 uptakes during the second six minute equilibration period were made. These corrections were determined by extrapolation from the first two manometer readings taken during the incubation. The temperature of each flask was assumed to be near enough to that of the bath to enable an accurate estimate of the O_2 uptake during equilibration. Any shock reaction of the enzyme particles, due to the sudden change in their environment when they are subjected to the Warburg flask contents and the bath temperature, would have time by this method to come to equilibrium.

In the second method the enzyme was added to a single Warburg flask containing the other ingredients of the reaction medium, the flask was swirled for 10 seconds, and an aliquot of 0.02 ml. was taken immediately for the Pi determination. This process was repeated for each flask in the experiment. All of the flasks were kept in an iced metal pan until all of the aliquots had been withdrawn. The flasks (on their manometers) were then placed on the Warburg bath in rapid succession and equilibrated 6 minutes before the manometers were closed. Again an extrapolation was made to determine the O_2 taken up during the six minute equilibration period. No correction was made for any phosphorylation during the 8 to 12

minute aliquoting time since the flasks were kept in the iced metal pan, and supposedly very little phosphorylation took place. At the end of the incubation period 0.02 ml. aliquots were again taken, using the same method as was described in the preceeding paragraph for taking final aliquots. This second method enabled a better control of the timing between Pi aliquots, but it suffered from the disadvantages of possible shock effects, first reading fluctuations caused by temperature differences, and temperature induced errors in the estimated O_2 uptake during equilibration.

PHOSPHORUS DETERMINATION

The method used for the determination of Pi was essentially the "isobutyl alcohol colorimetric method" developed by Pons, Stansburg, and Mack (36, p.495). An isobutyl alcohol extraction of the inorganic phosphomolybdate complex enabled the removal of organic phosphates. In this way a stable, blue colored solution was obtained upon addition of the stannous chloride reagent. Because of this, accurate timing between colorimeter readings was not necessary. A standard curve was determined each time the phosphorus method was used, some variation in standard curves being observed. This variation was probably caused by changes in the reagents used in the Pi determination. It was found that more reliable Pi

results were obtained from the Warburg flask contents when the concentration of stannous chloride, suggested by Pons, Stansburg, and Mack, was doubled. No explanation is suggested for this other than the possible interference of small amounts of the enzyme preparation. No trichloroacetic acid precipitation of proteins in the aliquots was necessary since clear isobutyl alcohol solutions were obtained.

RESULTS AND DISCUSSION

From the beginning of this study of oxidative phosphorylation, P:O ratios above 1.0 could routinely be obtained. Having this Pi uptake from the outset of this work greatly facilitated the study of the cofactors and conditions necessary for obtaining optimal phosphorylation.

CONDITIONS FOR OPTIMAL PHOSPHORYLATION

Sucrose concentration

The sucrose concentration in the Warburg flasks was varied. Slightly higher oxidative phosphorylation activity was found when the medium contained 0.375 M sucrose than when it contained 0.625 M sucrose.

Versene

In experiments where the concentration and presence or absence of Versene (ethylenediaminetetracetate) was

varied in the blending, washing, or final suspension mediums, slightly better oxidative phosphorylationwas obtained when 0.01 M Versene was included only in the homogenizing mediums. This effect of Versene could be reversed completely when 0.005 M $CaCl_2$ was added.

It was observed that Versene produced variable effects on the O_2 uptake obtained from different substrates. When 0.035 M Versene and 0.1 M phosphate were used as the only solutes in the homogenizing, washing and suspension mediums, the net O_2 uptake was 55 µA. for malate, 46 µA. for pyruvate, and 12 µA. for citrate. The net O_2 uptake obtained for the same amounts of substrates using the usual mediums in the preparation was 32 µA. for malate, 28 µA. for pyruvate, and 90 µA. for citrate. It appears from these results that sucrose is necessary for maximal citrate oxidation, but that Versene in lower concentration can be substituted for the sucrose to give better oxidation of malate and pyruvate.

Glucose

An experiment was conducted in which the subcell particles for half of the Warburg flasks were obtained using 0.5 M glucose, 0.1 M sucrose, and 0.1 M phosphate buffer for the homogenizing, washing and suspension mediums. The usual preparation, in which the medium contained 0.6 M sucrose and 0.1 M phosphate, was introduced into the other Warburg flasks. It was found that

a net average O_2 uptake of 532 µl. was obtained from α-ketoglutarate when the glucose-sucrose-phosphate medium had been employed, while an average uptake of 611 µl. was obtained when the sucrose-phosphate medium had been used. Lower concentrations of glucose in the Warburg flask were tested. 0.1 M glucose did not cause as great a reduction in O_2 uptake, so it was selected as the concentration to be used in later phosphorylation experiments.

Hexokinase

The effect of a hexokinase enzyme concentrate from yeast was tested in a number of experiments. Hexokinase was added to certain Warburg flasks and omitted from other flasks in the same experiment. Table 7 shows the results obtained from three of the experiments.

As can be seen in Table 7, variable results were obtained from the addition and omission of hexokinase from the reaction mixture. In some cases the hexokinase preparation caused a reduction in the P:O ratio and in other experiments it caused an increase in the P:O ratio. These results can be explained if one assumes that the exact conditions for the preparation of the particulate enzyme concentrate were not the same in the different experiments. In one case the blendor might disorganize the particles to such a degree that most of the hexokinase normally present is lost. In this case only low P:O

Table 7

The effect of the omission of hexokinase on oxidative phosphorylation by subcell particles from cabbage

Experiment, incubation time, substrate and amount added	Additions	Endogenous O_2 uptake	Net substrate O_2 uptake	Endogenous P uptake	Substrate P uptake	P:O ratio
		µA.	µA.	µA.	µA.	
1. 120 min.	Complete[a]	9.6	50	9.0	96	1.9
α-ketoglutarate 20 µM.	Complete-hexokinase	10.0	59	9.0	43	0.73
2. 60 min.	Complete	2.1	12.3	-12	34[b]	2.8
α-ketoglutarate 10 µM.	Complete-hexokinase	-	17.3	-	12.5[b]	0.72
3. 50 min.	Complete	2.0	16.1	- 6	49.3[b]	3.0
α-ketoglutarate 10 µM.	Complete-hexokinase	-	11.2	-	42.5[b]	3.8

[a] The complete system is described under "PREPARATION, SUSPENSION, AND ANALYSIS."
[b] These values are not corrected for the endogenous phosphorus fixation.

ratios would be obtained in the absence of added hexokinase. With other preparations the condition of the plant material might be such that facile blending can be accomplished. In this case little disorganization of the particles might occur, and therefore maximum P:O ratios could be obtained without added hexokinase. The author observed a considerable variation in the ease of blending material from two different cabbage heads of approximately the same size and harvested at the same time.

As was mentioned in the introduction of Part II of this thesis, the preparations of Bonner and Millerd, and of Millerd, did not require external hexokinase for phosphorylation to occur (8,pp.141-142; 32, p.860). It should be mentioned that the greatest phosphorylation effect reported in these articles involved the uptake of only 3 μA. of Pi.

If one assumes an incubation period of 20 minutes for Laties' phosphorylation experiments (26, p.568) then it can be calculated (from data presented by him) that in some of his better experiments 12.0 μA. of Pi were taken up with every 5 μA. of oxygen. Laties added a yeast hexokinase preparation to his reaction flasks.

Conn (15) reported verbally at the April 11-15, 1955 meeting of the Federation of American Societies of Experimental Biology that lupine mitochondria were able

to incorporate as much as 15 µmoles of Pi into hexose phosphate without the addition of external hexokinase.

It is not surprising, in view of these findings by other workers, that added hexokinase was partially required for the phosphorylations reported in Table 7, since usually more than 20 µM Pi were incorporated into organic compounds. In some cases as much as 100 µmoles of Pi were incorporated.

Fluoride

The fluoride concentration was varied in a number of experiments. 0.01 M NaF caused a 5 to 10% reduction in oxidation and phosphorylation when the results were compared to those obtained from flasks containing no NaF. When 0.02 M NaF was used, a much larger reduction in O_2 uptake took place; however, a slight increase in P:O ratio was obtained. At higher concentrations of NaF, a very large reduction in O_2 uptake was obtained along with low P:O ratios.

Phosphate concentration

The phosphate concentration necessary for maximal oxidation and phosphorylation was tested. At concentrations of phosphate below 0.0625 M, results were obtained that were similar to those reported by Beaudreau (3, pp.58-60). When the number of micromoles of phosphate per flask was reduced from 200 to 100 no significant reduction in oxidative activity was observed during the

first 60 minutes. However, it should be remembered that if 50 μmoles of Pi are fixed in thirty minutes then the phosphate concentration could become limiting and a reduction in oxidative phosphorylation would occur. It is for this reason that 100 to 200 μmoles of phosphate per flask were used in the phosphorylation experiments. When phosphate concentrations higher than 0.0625 M (200 μmoles/flask) were used no significant loss or gain in P:O ratio was obtained.

pH

The pH in the Warburg flask did not seem to be too critical provided that it was kept in the range from 6.7 to 7.1. The optimal pH varied with the substrate used and with the individual cabbage particulate preparation. Generally it can be stated that the best substrate induced O_2 uptake and phosphorylation was obtained at or near pH 6.8.

Cofactors

The cofactors found necessary for the highest substrate induced O_2 uptake and phosphorylation were DPN, TPN, Mg, Mn, ATP, and GSH. Some of the results used to determine the required cofactors are shown in Table 8.

Omission of manganese did not cause any change in the first hour rate of oxygen uptake, but it did cause a pronounced reduction in the amount of Pi fixed as organic phosphate. This suggests a possible role of manganese in phosphorylation.

Table 8

The effect of various cofactors on the oxidative activity of subcell particles from cabbage

Experiment number	Period of incubation	Additions	O_2 uptake	
			Endogenous	Malate induced
			µl.	µl.
4.	6 hrs.	complete[a]	183	300
		complete-DPN, TPN, TPP, and liver concentrate	120	83
		complete-Mg and Mn	130	153
		complete-ATP	101	148
6.	6½ hrs.	complete	177	433
		complete-cytochrome c	182	475
		complete-GSH	198	250
		complete-liver concentrate	176	532
		complete-TPP	185	499

[a] The complete system is the same as that listed under "PREPARATION, SUSPENSION, AND ANALYSIS" except for the omission of NaF, glucose, and hexokinase, and the addition of 1.4×10^{-3} M TPP and 0.125 mg liver concentrate per ml.

Variable results were obtained with cytochrome c. Usually added cytochrome c was not required, and even better O_2 uptake and P:O ratios were obtained in its absence. In a few experiments the best O_2 uptake and phosphorylation took place when cytochrome c was added, but in these experiments low P:O ratios were obtained. It is therefore suggested that intact particles contain sufficient cytochrome c, or a natural substitute, and that the higher P:O ratios were obtained with such particles. The lower P:O ratios were probably obtained with partially damaged particles, which could not retain their endogenous cytochrome c or a natural substitute. Thus, the cytochrome c requirement of the particles probably gives an indication of the degree of particulate organization. This same suggestion has also been made by Millerd (32, p.859).

In a number of experiments no effect was produced when the Co A concentrate and TPP were added. These two substances do not seem to be required.

Phosphatases and anaerobic phosphorylation

Experiments were conducted to determine the extent of phosphatase hydrolysis of organic phosphate. The determinations of the amounts of O_2 and Pi uptake were carried out in the usual manner. The flask contents contained all the additives described under "PREPARATION, SUSPENSION, AND ANALYSIS" with the exception of

hexokinase, fluoride, glucose, and sucrose. Mannitol was substituted for the glucose and sucrose, and no TCA cycle substrate was added. An addition of 110 µmoles of ATP was made in place of the 10.3 µmoles usually present in each Warburg flask. On an average, 8 µmoles of Pi were liberated in 70 minutes in such experiments. This was associated with an average O_2 uptake of 3 µatoms. These results suggest that a slight amount of phosphatase activity was present in the preparations. When the endogenous phosphorus exchange data obtained in a number of the usual experiments were compared, a wide variation was noted. It was not uncommon to find that all of the 10.3 µmoles of ATP added were hydrolized, but this was not always the case. In addition the Pi exchange results from duplicate "endogenous" flasks frequently were not in good agreement. Because of this and becasue of the limited accuracy of the Pi determination method, the substrate induced Pi uptake values given in this thesis usually are not corrected for the endogenous phosphorus exchange.

When nitrogen was substituted for the air atmosphere in the reaction flasks, no Pi was taken up and no change in the manometer readings occurred. This indicates that the Pi uptake into organic form is associated with the O_2 uptake.

Malonate

Although 0.01 M malonate caused, in a number of experiments, an average inhibition of 90% in the O_2 uptake induced by 10 μM of succinate and an average inhibition of 48% in the O_2 uptake induced by 10 μM of α-ketoglutarate, this inhibitor did not significantly change the P:O ratios obtained with these two substrates. Therefore, malonate was not added routinely in the phosphorylation experiments.

Concentration of subcell particles

Table 9 shows that the concentration of subcell particles is an important factor for obtaining maximum P:O ratios. As the particulate enzyme concentration was reduced, the efficiency of phosphorylation, or the P:O ratio, was consistently increased. It is difficult to give an explanation of this effect. Several ideas were tested, but none seemed to explain the results.

Incubation period

It is to be noted that the highest P:O ratio shown in Table 9 (see experiment 5) was obtained using the shortest incubation period. This relationship was observed frequently in experiments not reported here. That is, short incubation periods generally led to higher P:O ratios than did longer periods. This is not surprising since it is reasonable to assume that the phosphorylating system would suffer damage with prolonged incubation

Table 9

The effect of enzyme concentration on P:O ratios[a]

Experiment number and incubation time	Weight of soluble cabbage represented[b]	Endogenous Pi uptake	Substrate induced Pi uptake[c]	Endogenous O_2 uptake	Net substrate induced O_2 uptake[d]	P/O
	gm.	µA.	µA.	µA.	µA.	
1. 60 min.[e]	11.7	-11.0	52.4	5.0	25.2	2.1
	23.4	2.0	47.8	7.2	30.0	1.6
	35.1	- 8.7	29.5	8.0	31.3	0.9
2. 50 min.	11.9	-	64.0	-	24.2[f]	2.64
	23.8	-	63.5	-	30.0[f]	2.12
	35.7	-	60.4	-	31.9[f]	1.89
	47.6	-	48.6	-	34.0[f]	1.43
3. 51 min.	13.5	- 0.8	27.0	2.0	9.0	3.0
	26.5	- 6.3	43.3	1.6	16.0	2.7
4. 60 min.[e]	48.0	3.7	70.1	3.3	23.3	3.0

Table 9 (Continued)

Experiment number and incubation time	Weight of soluble cabbage represented[b]	Endogenous Pi uptake	Substrate induced Pi uptake[c]	Endogenous O_2 uptake	Net substrate induced O_2 uptake[d]	P/O
	gm.	µA.	µA.	µA.	µA.	
5. 30 min.	16.4	-	24.4	6.9	6.4	3.8
	49.2	-	16.6	8.9	13.0	1.4

[a] Each value given is the average of that found for at least two flasks.
[b] This weight was determined as follows: The weight of the solid material remaining behind after the homogenate was filtered through the cheesecloth was subtracted from the total weight of plant tissue homogenized. The fraction of the total volume of the final particle suspension which was added to a given Warburg flask, multiplied by the weight of cabbage that was in the solutions centrifuged, gave this value.
[c] Not corrected for endogenous Pi uptake.
[d] The substrate used was α-ketoglutarate. No malonate was added in these experiments.
[e] The particulate preparation used in these experiments was washed twice.
[f] These values are not corrected for endogenous O_2 uptake.

at 30° C.

Incubation periods of 30 minutes or less were not used routinely in the phosphorylation experiments because of the variation in the endogenous Pi exchange and because of the limited accuracy of the Pi determination. It will be recalled that each Warburg flask contained approximately 200 μmoles of Pi at the beginning of the experiments. With this high level of Pi a rather large fixation of Pi was required to obtain reasonably accurate estimates of the P:O ratios. The longer incubation periods were generally required to obtain this large Pi fixation.

Condition of the plant material

The P:O ratios given in this thesis serve to indicate that reasonably good oxidative phosphorylation is catalyzed by the subcell particles from cabbage. However, it is suggested that the determination of highly accurate and truly representative P:O ratios would require rigid standardization of the plant material and the conditions for the preparation of the enzymes. The variation shown in Table 9 between individual experiments seemed to be largely dependent upon differences in the condition of the plant material. Better P:O ratios were obtained with easily homogenized plant material. The manner of preparation of the active fraction seemed to affect phosphorylation more than it did O_2 uptake.

Dialysis

A portion of the cabbage particulate preparation was tested for phosphorylation as usual. Another equal portion was dialyzed 24 hours at 0° to 5°. The dializing medium containing 0.6 M sucrose, 0.1 M phosphate buffer at pH 7.0, 6.1×10^{-4} M GSH, 4.8×10^{-6} M DPN, 4.3×10^{-6} M TPN, trace amounts of TPP and Co A, and four substrates (citrate, succinate, malate, and α-ketoglutarate), each at 1.3×10^{-5} M. After dialysis the enzyme preparation was tested for phosphorylation as usual. Dialysis caused a 39% reduction in the P:O ratio obtained in the usual manner. This effect included a 39% reduction in O_2 uptake and a 61% reduction in overall Pi uptake.

Other phosphate acceptors

When phosphocreatine (P-creatine), inosine triphosphate (ITP) and uridine triphosphate (UTP) were added to the reaction flasks in both the presence and absence of ATP, and with oxalacetate as the substrate, variable results were obtained. Table 10 shows the results obtained.

P-creatine had no significant effect on O_2 or Pi uptake in the presence of ATP. P-creatine in place of ATP could not support either oxidation or phosphorylation.

ITP in the presence of ATP was inhibitory to

Table 10

The effect of other phosphorus compounds on phosphorylation[a]

Additions	O_2 uptake μA.	P uptake μA.	P/O
Complete	25.7	34.7	1.3
Complete-ATP + P-creatine	10.2	0	-
Complete + P-creatine	25.5	28.0	1.1
Complete - ATP + ITP	18.5	3.7	0.2
Complete + ITP	25.9	0	-
Complete - ATP + UTP	24.0	16.9	0.7

[a] Each value given is the average of data obtained from duplicate Warburg flasks. The substrate used was oxalacetate (20 μmoles/flask).

Pi uptake. ITP could partially support O_2 uptake in the absence of ATP but it could not support phosphorylation.

UTP could partially replace ATP for both O_2 and Pi uptake.

PHOSPHORYLATION CATALYZED BY A NUMBER OF TCA CYCLE INTERMEDIATES

Table 11 shows that P:O ratios between 1 and 2 were obtained when glutamate and citrate were used as substrates. P:O ratios between 2 and 3 were obtained with oxalacetate, fumarate, and succinate, and P:O ratios above 3 were obtained only when α-ketoglutarate was the substrate. A variation in the P:O ratio supported by a given substrate was obtained between different experiments. The values given in Table 11 represent some of the better P:O ratios obtained for each substrate.

Inhibitors of TCA cycle enzymes were not used. For this reason the P:O ratios observed represent O_2 and Pi uptake from the operation of many steps in the TCA cycle rather than those associated with just a single oxidative step. The high P:O ratios obtained for α-ketoglutarate oxidation lead one to believe that at least a large share of the O_2 and Pi uptake during the first 50 minutes is associated with a single oxidative step in this case.

Table 11

The effect of the substrate upon phosphorylation[a]

Experiment number, incubation time, substrate used, and amount added	Pi uptake	O_2 uptake	P:O ratio
	µA.	µA.	
1. 50 min.			
α-Ketoglutarate, 10 µm.	42.5[b]	11.2[b]	3.8[b]
Oxalacetate, 20 µm.	49.8	22.4	2.2
Fumarate, 20 µm.	42.8[b]	18.7[b]	2.3[b]
Succinate, 10 µm.	23.6[b]	10.2[b]	2.3[b]
2. 30 min.			
α-Ketoglutarate, 20 µm.	26.8	7.0	3.8
3. 50 min.			
Citrate, 10 µm.	29.0	22.9	1.3
4. 60 min.			
Glutamate, 20 µm.	64.0[b]	37.0[b]	1.7[b]

[a] Each value given is the average of results from two duplicate flasks unless indicated otherwise.
[b] Results from one flask only.

SUMMARY

Phosphorylation of a variable nature has been shown to occur when cabbage particulate fractions are incubated in the proper medium with TCA cycle intermediates. P:O ratios as high as 3.8 have been obtained for the oxidation of α-ketoglutarate. P:O ratios for other TCA cycle intermediates were also determined.

The conditions necessary for optimal oxidative phosphorylation have been studied.

The magnitude of the Pi uptake is great enough to remove any doubt that oxidative phosphorylation is associated with the TCA cycle reactions in cabbage particles.

PART III EXTENT OF SUBSTRATE OXIDATION, LABELING OF TCA CYCLE INTERMEDIATES, AND OTHER REACTIONS RELATED TO THE TCA CYCLE

INTRODUCTION

The catalysis of the oxidation of endogenous compounds upon the addition of TCA cycle intermediates has been observed with many animal preparations. Krebs and Johnson (25, p.149) found that with minced pigeon breast muscle, citrate caused an extra O_2 uptake that was far greater than could be accounted for by complete oxidation of the citrate. Stare and Baumann (40, p.354) found that when very small amounts of fumarate were added to muscle preparations, O_2 uptake increased 6 to 10 times more than could be accounted for by the complete oxidation of the added fumarate. The catalysis of fatty acid oxidation by TCA cycle intermediates has been well verified. Lehninger and Kennedy (27, pp.753-771) have suggested an explanation for this catalysis of fatty acid oxidation. They found that small amounts of TCA cycle intermediates were required to initiate the formation of other TCA cycle members from the fatty acids.

A catalysis similar to any one of those mentioned above would completely invalidate the calculation of the percent oxidation of the substrates. For this reason a

study was undertaken to determine the actual percent of substrate oxidation. Succinic acid-2-C^{14} was used in this study.

Brummond and Burris (9, pp.754-759) demonstrated that lupine mitochondria could incorporate C^{14} from pyruvate-2-C^{14} into each of the major intermediates of the TCA cycle. This evidence shows that each major intermediate of the TCA cycle was derived in some manner from the labeled pyruvate and argues strongly for the complete operation of the TCA cycle.

Stumpf (41, p.256) found transaminase activity between α-ketoglutarate and alanine, aspartic acid, α-aminobutyric acid, leucine, isoleucine, valine and norvaline in dialized extracts of wheat germ, pumpkin leaves, lima bean seedlings, lupine seedlings, pumpkin seedlings, and pea seedlings. Wilson, King, and Burris (43, pp.863-874) observed the presence of transaminases in extracts from a number of plants which could transfer the amino group from each of seventeen different amino acids to α-ketoglutarate. Aspartic acid, alanine, serine, lysine, tyrosine, and arginine were a few of the amino acids found to be involved in this transamination.

Humphreys, et al (22, pp.941-948) obtained evidence for a simple decarboxylation of palmitic acid-1-C^{14} by the microsomal and supernatant fractions from peanut

cotyledons. This decarboxylation did not seem to take place via the TCA cycle oxidation of acetate, which was derived from the palmitic acid.

METHODS AND MATERIALS

CONDITIONS FOR MANOMETRIC MEASUREMENT OF PERCENT OXIDATION OF TCA CYCLE MEMBERS

The same methods and mediums were used for the preparation and incubation of the cabbage particles as those described under "METHODS AND MATERIALS" in Part I of this thesis. Larger amounts of enzyme than usual were added to a given Warburg flask to increase the rate of substrate oxidation.

METHODS USED FOR C^{14} EXPERIMENTS

Succinic acid-2-C^{14}, with a specific activity of 0.76 millicuries/millimole (0.76 mc/mM), was purchased from Tracerlab Incorporated. A 0.1 M solution of potassium succinate-2-C^{14} was prepared using only the labeled succinic acid. Eight 110 gm. portions of chopped cabbage were homogenized, strained, centrifuged, and suspended as described under "METHODS AND MATERIALS" in Part I of this thesis. The entire amount of particulate material obtained was suspended in 28 mls. of 1 M sucrose and 0.1 M phosphate buffer. Two mls. of this

suspension were added to each of 14 Warburg flasks containing the same reaction medium as described previously. The addition of 10 μmoles of succinate-2-C^{14} (0.1 ml. of 0.1 M Succinate-2-C^{14}) was made to three of the Warburg flasks. Three other Warburg flasks were used to determine the endogenous O_2 uptake. The eight remaining flasks were used to determine manometrically the CO_2 evolution from 10 μmoles of nonlabeled succinic acid by the "direct method" of Dixon and Warburg (42, pp.17-19). No folded paper squares were added to the center wells of any of the flasks. An addition of 0.2 mls. 2 N H_2SO_4 was made to the side arm of each of the 14 Warburg flasks. The total side arm acid of different flasks was tipped into the main compartment at various times to enable the evolution and the measurement of the CO_2 retained in the buffer. The Warburg flasks and manometers were incubated until close to 100% of the theoretical O_2 uptake, for complete oxidation of the succinate, had taken place.

A number of dry combustions of known amounts of unlabeled succinate were carried out by following the procedure suggested by Niederl and Niederl (34, pp.101-139). The CO_2 was collected and the $BaCO_3$ was precipitated as described by Calvin, et al (14, pp.84-88). An average of 103% of the theoretical weight of dry $BaCO_3$ was obtained. Four additional dry combustions were

conducted using varying known amounts of the 0.1 M succinate-2-C^{14} solution (0.01 ml. to 0.04 ml.) and nonlabeled carrier succinate. The total weight of the dried $BaCO_3$ was determined. An average recovery of 104% was obtained. A number of $BaC^{14}O_3$ planchets were prepared after each combustion of labeled succinate in the same manner as described by Cook and Duncan (17, pp.239-241). These were counted in the conventional manner, using a Geiger-Muller counter with a thin mica window; the data were corrected for background and self-absorption.

At the end of the $5\frac{1}{2}$ hour incubation of the Warburg flasks, the center well KOH was quantitatively removed from the flasks which orginally contained labeled succinate. A known weight of nonlabeled carrier carbonate was added to each (1.5 to 1.7 gms. anhydrous Na_2CO_3). $BaC^{14}O_3$ was precipitated and its total dry weight was determined as described previously. Again $BaC^{14}O_3$ planchets from each of the three samples were prepared, weighed, and counted.

The contents in the main compartments of the three labeled succinate flasks were quantitatively transferred, after the incubation, to three centrifuge tubes containing 12.8 mls. of absolute ethyl alcohol in each. The tubes were then centrifuged at 2,000 RPM for 10 minutes. The supernatant liquids were removed and each of the

protein precipitates was washed several times with 80% alcohol. The protein precipitates were then dried and weighed. A known portion was removed from each, combusted to CO_2, precipitated as $BaCO_3$, dried, weighed, plated, and counted as described previously. The supernatant fractions, including their respective wash solutions, were evaporated to a volume of approximately 2 ml. each.

A 0.03 ml. aliquot from each of the supernatant concentrates was chromatographed directly with known TCA cycle acids as described by Buch, Montgomery, and Porter (10, pp.489-491). Aliquots of 0.07 ml. from each of the concentrates were chromatographed directly with known amino acids using the procedure of Block, Le Strange, and Zweig (7, pp.51-54). With both chromatographic procedures a number of descending one dimensional chromatograms were prepared from each supernatant concentrate. The chromatograms were counted with a manual strip counter. Radioautographs were prepared from representative chromatograms using a procedure similar to that described by Katz and Chaikoff (23, pp.891-892). Exposure time was two months.

Palmitic acid-1-C^{14} (1 mc/mM) was purchased from Tracerlab Incorporated. A 0.001 M palmitate solution was prepared as described by Humphreys, et al (22,

p.942). An addition of 0.1 μmoles of palmitate with 10 μmoles of citrate was made to the usual reaction mixture in two duplicate Warburg flasks. The endogenous, palmitate, citrate, and palmitate-citrate induced O_2 uptakes were determined. At the end of the incubation period the center well contents were removed, prepared, and counted as described previously.

RESULTS AND DISCUSSION

PERCENT OXIDATION OF SUBSTRATES AS INDICATED BY O_2 UPTAKE DATA

The amount of O_2 uptake induced by citrate with a cabbage particulate preparation is shown in Figure 3. As 100% oxidation (as calculated from O_2 uptake data) was approached, the substrate concentration appeared to become limiting and the substrate induced O_2 uptake approached zero. Similar curves were plotted with data obtained using malate, succinate, and α-ketoglutarate. At first it was thought that these graphs gave evidence for the complete oxidation of these substrates. Results to be mentioned later in this thesis indicated that close to 75% of the added substrates were actually oxidized when the theoretical O_2 uptakes for complete oxidation of the substrates had been obtained. Nevertheless at this point the rates of O_2 uptake in the

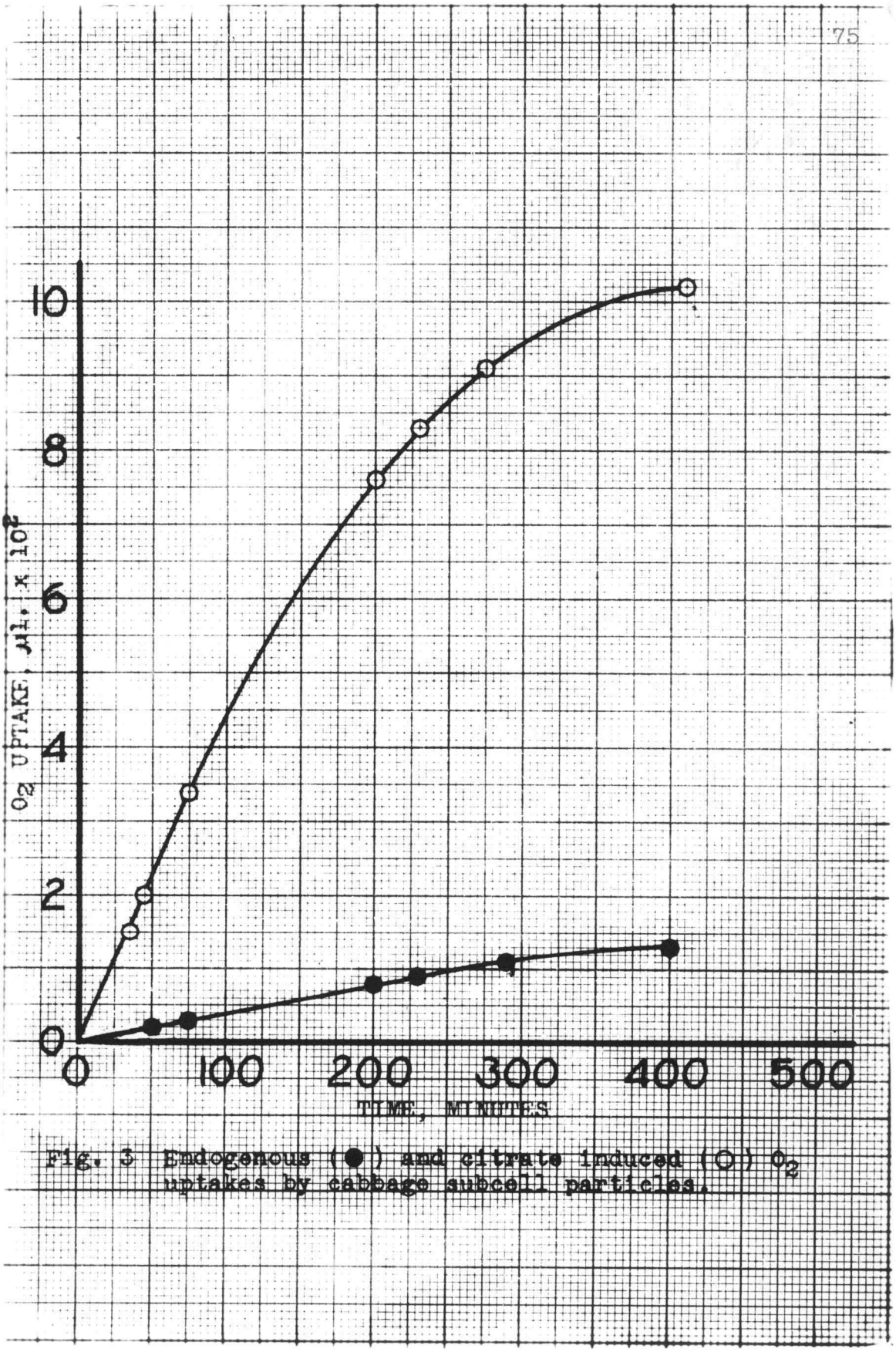


Fig. 3 Endogenous (●) and citrate induced (○) O_2 uptakes by cabbage subcell particles.

flasks containing substrates, were the same as the endogenous O_2 uptake rates. A typical endogenous O_2 uptake curve is included in Figure 3.

SUCCINATE-2-C^{14} EXPERIMENTS

Strip 1 in Figure 4 is a photograph of a chromatogram developed from known unlabeled TCA cycle intermediates and an aliquot from one of the three "labeled succinate" supernatant concentrates. Strip 2 is a radioautograph of strip 1. Strips 3 and 4 are radioautographs of chromatograms developed from aliquots taken out of the other two labeled succinate supernatant concentrates. Known TCA cycle intermediates were also cochromatographed with these aliquots. Strips 5, 6 and 7 are radioautographs of chromatograms developed in solvents for amino acid separation. Known amino acids were cochromatographed with aliquots from the three respective labeled succinate supernatant concentrates to obtain these three chromatograms. A smaller sized aliquot of the supernatant concentrate was used for strip 5.

The aliquots taken from the succinate-2-C^{14} supernatant concentrates did not have sufficient quantities of TCA cycle intermediates or amino acids (with the exception of glutamate) present to give color on the

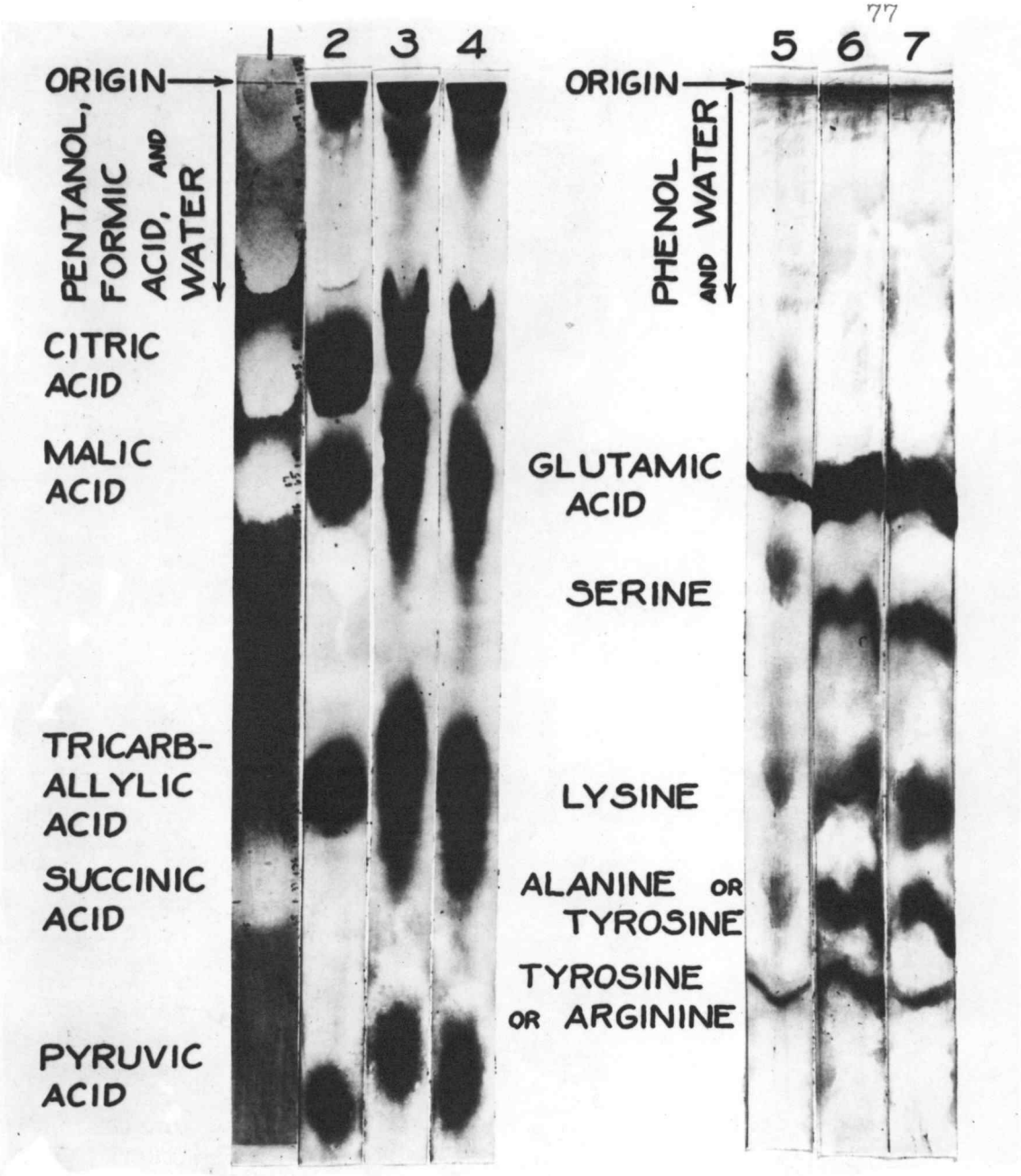


Fig. 4. Products formed from succinic acid-2-C^{14} by the cabbage particulate preparation. Strip 1 is a photograph of a chromatogram obtained from an aliquot of the soluble Warburg flask contents with known acids added. Strip 2 is a radioautograph of strip 1. Strips 3 and 4 are radioautographs of chromatograms made from aliquots taken from the soluble fractions of duplicate Warburg flasks. Strips 5-7 are radioautographs made from aliquots taken from the soluble fractions of triplicate Warburg flasks.

chromatograms when the usual color producing reagents were applied. A colored radioactive band was obtained, after ninhydrin treatment, which coincided with known glutamate. This indicated that relatively large amounts of labeled glutamate were synthesized from the succinate-2-C^{14} by the cabbage particles.

It should be noted that radioactivity from the succinate-2-C^{14} was incorporated into citrate, glutamate, malate, pyruvate, tricarballylate, serine, lysine, alanine or tyrosine, and tyrosine or arginine. This gave good supporting evidence for the operation of the complete TCA cycle in cabbage particles. Good evidence is also given for amino acid synthesis from TCA cycle intermediates and for transamination reactions in the cabbage particles.

It can be seen in Figure 4 that no labeled area was obtained on radioautograph 2 (strip 2) which coincided with the known succinic acid spot seen on the photograph (strip 1) of the same chromatogram.

Other chromatograms (not shown here) were obtained in which the known tricarballylic acid spots on the chromatograms coincided with the labeled areas found on their respective radioautographs. This chromatographic identification of tricarballylic acid was repeated many times. Maleic acid has a very similar R_f value to that

of tricarballylic acid, but the known maleic acid spots on the chromatograms did not coincide well with the labeled areas on the radioautographs. Neither tricarballylate or maleate caused an O_2 uptake when they were added individually to the reaction mixture either with or without other TCA cycle intermediates.

Little work has been done on the metabolism of tricarballylic acid in plants. Tricarballylic acid has been recognized as a by-product from the processing of sugar beets (29, pp.222-223). Nelson and Mottern (33, pp.3046-3048) found that tricarballylic acid could be isolated from barley and maize plants.

Table 12 shows the activity found to be present in 0.1 ml. of the 0.1 M succinate-2-C^{14} solution. This represented the total radioactivity added to each of the three Warburg flasks. The total activities found in the center wells and in the protein fractions after the incubation are also given in the table. It can be calculated that 73% of the radioactivity in the original succinic acid added to the Warburg flasks was recovered in the center wells as $C^{14}O_2$ when the O_2 uptake data indicated there was 98% oxidation of the added substrate. If labeled TCA cycle intermediates derived from the succinate-2-C^{14} had exchanged for nonlabeled intermediates, or with amino acids, then 98% oxidation could actually

Table 12

Total radioactivity added to the Warburg flasks and the total activity found in the center well KOH and protein fractions[a]

Fraction	c.p.m. x 10^5
Activity found present in 10 μM. of succinate-2-C^{14}	6.52[b]
Total activity recovered from center well KOH at 98% of the theoretical O_2 uptake	4.75[c]
Total activity in the protein fraction at 98% of the theoretical O_2 uptake	.008[c]

[a] The total incubation time was 5½ hours.
[b] Average of four determinations.
[c] The average of three triplicate Warburg flasks.

have taken place.

The labeled glutamate and the other labeled amino acids, as indicated in Figure 4, suggest the formation of amino acids from labeled carbon compounds by means of transamination reactions. Labeled α-ketoglutarate could transaminate with nonlabeled amino acids. By this means nonlabeled keto acids would be introduced into the TCA cycle for oxidation in place of the labeled α-ketoglutarate. It seems reasonable to conclude from this that the total oxidation of carbon compounds exceeded the equivalent of 73% of the original TCA cycle intermediate added. The manometric CO_2 data from the experiment indicated that approximately 98% of the CO_2 available from the succinate was liberated when 98% of the theoretical O_2 uptake was achieved. This CO_2 arose from succinate, exchanged substrates, and endogenous respiration. As indicated previously 73% of the CO_2 was derived from the succinate-2-C^{14}. This indicates that 25% of the CO_2 came from endogenous and exchanged substrates.

TRANSAMINATION

It was found that the cabbage particulate preparation catalyzed an O_2 uptake when either glutamate or oxalacetate was added to the reaction medium. Table 13, experiment 1, shows that small amounts of oxalacetate

were able to catalyze the oxidation of glutamate. This effect can be explained if glutamic-oxalacetic transaminase was present in the cabbage particles. When oxalacetate was present in the reaction medium with glutamate, transamination could take place to give aspartate and α-ketoglutarate. The α-ketoglutarate could then be oxidized through succinate to oxalacetate, which could then act as an additional acceptor for transamination. It is shown in Table 13, experiments 3 and 4, that when α-ketoglutarate was added with malonate a 17% inhibition of the O_2 uptake took place. When malonate was added with glutamate, a 68% inhibition in O_2 uptake took place. Malonate is considered to be a competitive inhibitor for the succinic dehydrogenase step. Therefore most of the malonate inhibition of glutamate oxidation could be explained as being due to a blocking of reactions necessary to furnish a transamination partner for the glutamate. Beaudreau (3, pp.17-23) found that these same transamination reactions took place in particles from Black Valentine beans.

The transamination data given in Table 13 give an explanation of why relatively large amounts of labeled glutamate were found to be derived from succinate-2-C^{14}. For optimal glutamate induced respiration a transamination keto acid partner is required. If a nonlabeled

Table 13

O_2 uptake data indicating transaminase activity in the subcell particles[a]

Experiment number, incubation time, substrates added, and μmoles added	Substrate induced O_2 uptake μA.	P:O
1. 150 Minutes		
α-Ketoglutarate 20	29.4	-
Oxalacetate 4, glutamate 20	43.4	-
Glutamate 20	18.4	-
α-Ketoglutarate 20, oxalacetate 4	26.2	-
2. 120 Minutes		
Oxalacetate 10	51.5	-
Glutamate 10	53.6	-
Glutamate 10, oxalacetate 10	64.2	-
α-Ketoglutarate 10	62.0	-
Succinate 10, oxalacetate 10	75.6	-
Succinate 10	69.2	-
3. 60 Minutes		
Oxalacetate 20	25.7	1.3
Glutamate 20, malonate 32	12.0	No Pi uptake
Glutamate 20	37.0	1.7
4. 60 Minutes		
Malonate 32, α-ketoglutarate 10	12	No Pi uptake
α-Ketoglutarate 10	14.4	3.24

[a] Each value given is the average of data from two duplicate Warburg flasks.

amino acid were to be oxidized via the TCA cycle, then some keto acid must be present for transamination since the results can not be explained completely by oxidative deamination. This transamination would then remove the keto acid from the oxidative reaction sequence. In the succinate-2-C^{14} experiments, this type of reaction would remove C^{14} labeled α-ketoglutaric acid from the substrate pool and would substitute unlabeled keto acids in its place.

Oxalacetate inhibition of succinate and malate oxidation with plant mitochondria has been reported by other workers (9, p.756; 3, pp.17-26). These workers could not obtain an oxalacetate induced O_2 uptake with their plant preparations. Beaudreau reported that 2 μM of oxalacetate caused an 88% inhibition of the O_2 uptake induced by 50 μM of succinate (3, p.25). Experiment 2, Table 13 indicates that at the most a 65% inhibition of the oxidation of 10 μmoles of succinate was caused by 10 μmoles of oxalacetate. The cabbage particles used in this work were able to oxidize oxalacetate. This lack of pronounced oxalacetate inhibition of succinate oxidation with the cabbage particles and the oxalacetate oxidation could indicate that the cabbage mitochondria have a high degree of integrity. Because of this the oxalacetate would have difficulty in getting to the

proper sites for inhibition. With a high degree of organization, oxalacetate could be oxidized more readily in a manner similar to the oxidation of other TCA cycle intermediates.

FATTY ACID OXIDATION

It was found that between 10 and 40% of the radioactivity added as palmitate-1-C^{14} was recovered as $BaC^{14}O_3$ after 6 hours incubation in the reaction mixture. The presence of palmitate in the reaction flasks had no effect on the rates of O_2 uptake. This could either indicate that fatty acid oxidation is associated with the cabbage mitochondria or that possibly microsomes were carried along with the particulate preparation. If this is microsomal activity, one would expect it to be similar to the simple decarboxylation reported by Humphreys, et al (22, pp.941-948).

SUMMARY

The theoretical amounts of oxygen uptake for complete oxidation of added succinate, malate, citrate and α-ketoglutarate were obtained with cabbage preparations.

Experiments involving the use of succinate-2-C^{14} indicated that 73% of the succinate was oxidized when 98% of the theoretical O_2 uptake and CO_2 evolution, for

complete succinate oxidation, was measured.

Cabbage preparations incorporated C^{14} from succinate-2-C^{14} into malate, pyruvate, citrate, tricarballylate, and glutamate. This gave additional evidence for the operation of the TCA cycle in the cabbage particulate preparations.

Results suggested that an exchange of endogenous compounds for TCA cycle intermediates took place during substrate oxidation experiments.

Aspartic-glutamic transaminase, as well as other transaminases, appear to be associated with the cabbage particles. Small amounts of serine, lysine, alanine or tyrosine, and tyrosine or arginine were synthesized from TCA cycle intermediates by the cabbage subcell particles.

The cabbage preparations catalyzed the formation of $C^{14}O_2$ from palmitate-1-C^{14}.

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