ENZYMIC ACTIVITY OF PARTICLES ISOLATED FROM VARIOUS TISSUES OF THE PEA PLANT

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Summary

A method is described for the preparation from green and etiolated tissues of the pea plant, of cytoplasmic particles with high oxidative activities in the presence of intermediates of the tricarboxylic acid cycle. The cofactor requirements have been investigated, and for preparations from etiolated pea stems, a phosphorylative ability comparable with animal mitochondria has been obtained. With certain of these preparations an oxidation of butyrate has been demonstrated.

I. INTRODUCTION

It has been shown that particulate preparations obtained from certain plant tissues contain an integrated system of enzymes, which can bring about the oxidation of intermediates of the tricarboxylic acid cycle and the subsequent synthesis of organic phosphate, the latter made possible by part of the energy released by these oxidations (Millerd *et al.* 1951). These particles were identified with the mitochondria present in the cytoplasm of intact plant cells. While cytological evidence has demonstrated the wide distribution of mitochondria in plant cells (see Newcomer 1951), the biochemical evidence of their ability to oxidize respiratory intermediates via the tricarboxylic acid cycle has been confined to relatively few tissues. Particulate preparations with this ability have been isolated from etiolated seedlings of mung bean (Millerd *et al.* 1951), pea (Davies 1953; Price and Thimann 1954), and lupin (Brummond 1952), the *Arum* spadix (Hackett and Simon 1954), potato tubers (Sharpensteen and Conn 1954), cauliflower buds (Laties 1953*a*), and the fruit of the avocado (Millerd, Bonner, and Biale 1953), and apple (Pearson and Robertson 1954). Little is known of the biochemical properties of such particles occurring in roots, while Brummond (1953) has cast doubt on the localization of the tricarboxylic acid cycle, as an integrated system, in cytoplasmic particles in the green leaves of lupins.

The purpose of this investigation was to demonstrate that biochemically active mitochondria could be isolated from various tissues of the one plant.

The results show that active mitochondria can be isolated from all major organs of both green and etiolated pea plants.

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II. Methods

(*a*) *Plant Material*

(i) *Germinated Pea Seeds.*—Pea seeds *Pisum sativum* (Yates Greenfeast) were soaked in water for 4 hr, then in mercuric chloride (0·001M) for 2 min. The seeds were well rinsed with water, placed on moist cotton wool and kept for 2 days in the dark at 20-25°C. For each preparation 60 g of material was used.

(ii) *Pea Seedlings.*—Pea seeds were pretreated with water and mercuric chloride as described above, then planted in moist vermiculite. Etiolated seedlings were used after 1-2 weeks growth at 20-25°C, while green seedlings were grown at the same temperature in sunlight and used after 2-3 weeks growth. For each preparation 80 g of etiolated stems, 60 g of roots or green stems, 10 g of green leaves, or 5 g of etiolated leaves were used.

(iii) *Peas.*—These were obtained from local markets, 50-60 g being employed for each preparation.

(*b*) *Chemicals and Enzymes*

Coenzyme concentrate (Coe) was made from dried Brewer's yeast by adsorption of the coenzymes from an aqueous extract onto charcoal, followed by elution with 10 per cent. pyridine and precipitation with acetone, according to the method of Le Page and Mueller (1949). The Coe contained 15·5 per cent. diphosphopyridine nucleotide (DPN) and 0·8 per cent. triphosphopyridine nucleotide (TPN), these substances being assayed by the methods of Racker (1950) and Le Page and Mueller (1949) respectively. DPN (50 per cent.), TPN (30 per cent.), and glucose-6-phosphate dehydrogenase were prepared as described by Le Page and Mueller (1949), alcohol dehydrogenase according to Racker (1950),* and hexokinase according to Berger *et al.* (1946). *Iso*citrate was prepared by hydrolysis of the lactone (Krebs and Eggleston 1944). Ethylenediaminetetraacetate, adenylic acid (AA), and other substances employed, were obtained from commercial sources.

(*c*) *Preparation of Isolated Particles*

With the exception of pea shells, in which a Waring Blendor was employed, the plant tissue was ground in a mortar with 1/5 vol. of sand and 1/3 vol. of sucrose (0·5M) containing ethylenediaminetetraacetate (5×10^{-3}M), pH 7·3. The brei was strained through muslin and centrifuged at 1200 *g* for 3 min. The supernatant was further centrifuged at 15,000 *g* for 10 min. After decantation the residue was resuspended in 80 ml of the extraction medium and recentrifuged at 15,000 *g* for 10 min. The washing procedure was repeated using sucrose (0·5M) containing 10^{-3}M ethylenediaminetetraacetate. Finally the residue was resuspended in a minimum volume of sucrose (0·5M). 0·6 ml of this preparation contained from 0·4-0·8 mg total nitrogen. All steps of the preparation were carried out at 0-5°C.

* The TPN and alcohol and glucose-6-phosphate dehydrogenases were kindly supplied by Dr. G. Kellerman.

(*d*) *Measurement of Activity*

The oxygen consumption was measured using Warburg manometers, all experiments, unless otherwise stated, being conducted at 30°C. Except where phosphate uptake was also estimated, the substrate was placed in the main compartment and readings commenced after an initial equilibration period of 5 min. For the measurement of phosphate uptake the substrate was tipped after 7 min and oxygen uptake readings taken between 7 and 37 min. The reaction was stopped by tipping 0·2 ml of 40 per cent. $HClO_4$ from a second side arm. The reaction mixtures, including controls which contained no substrate, were quickly chilled, centrifuged, and the inorganic phosphate present in the supernatant estimated by the method of Allen (1940).

TABLE 1

THE EFFECT OF ETHYLENEDIAMINETETRAACETATE IN THE EXTRACTION MEDIUM ON THE OXIDATIVE ABILITY OF PARTICLES FROM VARIOUS TISSUES OF THE PEA PLANT

Reaction mixture contains enzyme (0·6 ml), sucrose (0·4M), $MgSO_4$ (0·001M), AA (0·0005M), Coe (1 mg), phosphate (0·01M), and α-ketoglutarate (0·02M). Final volume 2 ml

Material	Oxygen Uptake* ($\mu l O_2$/mg N/30 min)	
	Extraction Medium	
	Sucrose (0·5M)	Sucrose (0·5M) containing Ethylenediaminetetraacetate (5×10^{-3} M)
Green leaf	98	117
Green stem	117	230
Root	18	60
Etiolated leaf	128	262
Etiolated stem	36	295
Germinated seed	72	146
Fresh pea seed	180	211
Hull	82	180

* Corrected for endogenous oxygen uptake.

III. RESULTS

(*a*) *The Oxidation of α-Ketoglutarate*

The oxidative abilities of particulate preparations obtained from the various parts of the pea plant tested are shown in Table 1. Since it was thought that substances contained in the vacuole might have some injurious effect on the enzymic capacity of the particles, ethylenediaminetetraacetate was added in the hope that it might protect the particles from some of these substances (cf. Slater and Cleland 1953). Results showed that this procedure increased the activity in every case and ethylenediaminetetraacetate was hence included in the extraction medium for all subsequent experiments.

(*b*) *Cofactors*

The cofactor requirements for the various preparations are shown in Table 2. Magnesium ions and AA are essential if good activities are to be obtained. In general, cytochrome *c* and DPN have little, if any, effect on the activity, but the coenzyme concentrate from yeast stimulates the rate of oxidation. In the case of etiolated stems, this stimulation is not due to DPN or TPN (Table 3).

(*c*) *Oxidative Phosphorylation*

The requirement for AA suggests that phosphorylation concomitant with the oxidation is taking place and this was verified directly for etiolated pea stems and green leaves by measuring the uptake of inorganic phosphate during active oxidation (Table 4). Hexokinase and glucose were used to 'trap' the

TABLE 2

COFACTOR REQUIREMENTS FOR THE OXIDATION OF α-KETOGLUTARATE BY PARTICLES FROM VARIOUS TISSUES OF THE PEA PLANT

The complete system contains enzyme (0·6 ml), sucrose (0·4M), phosphate (0·01M), $MgSO_4$ (0·001M), AA (0·0005M), cytochrome *c* (0·000005M), and α-ketoglutarate (0·02M). Final volume 2 ml

Material	Oxygen Uptake* (μl O_2/mg N/30 min)					
	-AA	-Mg	-Cytochrome *c*	Complete	Complete + DPN (0·001M)	Complete + Coe (1 mg)
Green leaf	22	19	140	139	137	221
Green stem	21	75	171	177	171	235
Root	28	14	49	47	40	244
Etiolated leaf	33	33	162	167	233	262
Etiolated stem	266	193	304	304	309	354
Germinated seed	27	31	59	81	79	111
Fresh pea seed	47	28	106	107	108	112

* Corrected for endogenous oxygen uptake.

esterified phosphate. Since the points of localization of phosphate esterification during the electron transport have not been determined, it is interesting to note that a phosphorylation also occurs when reduced DPN or TPN is used as substrate (Table 5). This was demonstrated by the addition of excess amounts of enzyme systems that reduce DPN and TPN respectively, namely the alcohol dehydrogenase and glucose-6-phosphate dehydrogenase systems.

(*d*) *The Oxidation of Other Substrates of the Tricarboxylic Acid Cycle*

With all the tissues tested, particulate preparations can be prepared, which will bring about the oxidation of pyruvate, *iso*citrate, glutamate, α-ketoglutarate, succinate, and malate (Table 6).

(e) *The Oxidation of Butyrate*

The oxidation of a fatty acid has not previously been demonstrated in plant mitochondria and the apparent lack of a system oxidizing fatty acids, ultimately via the tricarboxylic acid cycle and the cytochrome system, is the only major difference between mitochondria from animal and plant sources. Table 7 shows that butyrate can be oxidized by certain parts of the pea plant (green seedlings were not tested), but the oxidation is not easy to demonstrate. This is because of the apparent instability of the system. While the initial rate of

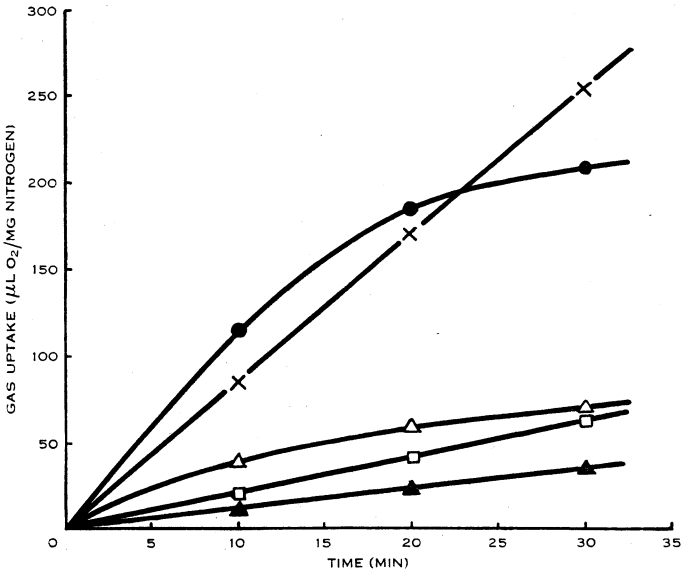

Fig. 1.—Rate of butyrate oxidation by particles from etiolated pea seedlings. Curves show the rate of oxygen uptake in the absence of added substrate (▲), with DL-malate (0·003M) (□), butyrate (0·05M) (△), DL-malate (0·003M) + butyrate (0·05M) (●), and α-ketoglutarate (0·02M) (X). Reaction mixture also contains enzyme (0·6 ml), sucrose (0·5M), phosphate (0·01M), $MgSO_4$ (0·001M), and AA (0·0005M).

oxidation is high (comparable to that of α-ketoglutarate), the rate soon falls to the level of the sparker oxidation, usually within 15 min and at the most 25 min. A typical rate curve for butyrate oxidation with particles isolated from the aerial portion of etiolated pea seedlings is shown in Figure 1. It can be seen that the presence of a small amount of tricarboxylic acid cycle substrate (in this case malate), greatly enhances butyrate oxidation.

(f) *The Duration of the Oxidative Ability of the Particles*

The oxidation of succinate, α-ketoglutarate, *iso*citrate, and glutamate was usually linear for at least one hour and in some cases for 2-3 hr. Notable

TABLE 3

THE EFFECT OF DPN, TPN, AND COE ON THE OXIDATION OF α-KETOGLUTARATE: ETIOLATED PEA STEMS

The reaction mixture also contains enzyme (0·6 ml), sucrose (0·4M), phosphate (0·01M), $MgSO_4$ (0·001M), AA (0·0005M). Final volume 2 ml

Reaction Mixture	Oxygen Uptake (μl O_2/mg N/30 min)
Enzyme	2
Enzyme + α-ketoglutarate	282
Enzyme + α-ketoglutarate + DPN (0·001M)	301
Enzyme + α-ketoglutarate + TPN (0·001M)	282
Enzyme + α-ketoglutarate + DPN + TPN	305
Enzyme + α-ketoglutarate + Coe (1 mg)	384

exceptions were the preparations obtained from root material, where the oxidation of all substrates was not linear and had generally ceased in 20 to 40 min. Further, the oxidation of pyruvate was not always linear and in most prepara-

TABLE 4

THE P : O RATIO FOR α-KETOGLUTARATE AND SUCCINATE

The reaction mixture contains enzyme (0·6 ml), sucrose (0·4M), glucose (0·02M), phosphate (0·04M), $MgSO_4$ (0·001M), AA (0·0005M), Coe (1 mg), and substrate (0·02M). Final volume 2 ml

Preparation	Number	Light or Dark	Substrate	Additions	Phosphorus Uptake (atoms × 10^{-6}/30 min)	Oxygen Uptake (atoms × 10^{-6}/30 min)	P : O
Etiolated pea stems	1		α-Ketoglutarate	—	0·0	12·3	—
	1		α-Ketoglutarate	Fluoride (0·1M)	1·9	4·0	0·47
	2		α-Ketoglutarate	Hexokinase (0·2mg)	36·1	12·6	2·86
	3		α-Ketoglutarate	Hexokinase (0·2 mg)	50·1	16·2	3·10
	4		Succinate	Hexokinase (0·2 mg)	35·5	24·5	1·45
	5		Succinate	Hexokinase (0·2 mg)	30·2	24·8	1·22
Green leaves (temp. 15°C)	6	Light*	Succinate	Hexokinase (0·2 mg)	11·9	8·4	1·42
	6	Dark	Succinate	Hexokinase (0·2 mg)	11·0	7·7	1·48

* Under the conditions of light and temperature used in this experiment, similar preparations from green pea leaves gave a photolytic activity of 600-800 μl O_2/mg chlorophyll/hr, using the ferricyanide assay method of Arnon and Whatley (1949).

tions fell off with time (cf. Laties 1953*b*). Where tried, the oxidation of citrate and fumarate proceeded at a rate comparable with that of glutamate.

IV. Discussion

Studies on the oxidative ability of isolated cytoplasmic particles from pea plants have been confined to the stems of etiolated plants (Davies 1953; Price and Thimann 1954). The activities reported here compare more than favourably with those previously obtained, but under the conditions used here, high activities could be obtained only if ethylenediaminetetraacetate was included in the extraction medium. A similar, though less striking effect, was obtained in all other tissues tested, and it would appear likely that the ethylenediaminetetraacetate protects the particles from some injurious substances, released from the cell vacuole.

Table 5

OXIDATIVE PHOSPHORYLATION WITH DPN AND TPN: ETIOLATED PEA STEMS

Substrate	Phosphorus Uptake (atoms $\times 10^{-6}$/30 min)	Oxygen Uptake (atoms $\times 10^{-6}$/30 min)	P : O
DPN*	5·2	5·3	0·98
TPN†	7·2	13·8	0·57

* Complete reaction mixture contains enzyme (0·6 ml), sucrose (0·4M), phosphate (0·02M), glucose (0·02M), hexokinase (0·2 mg), alcohol (0·2M), alcohol dehydrogenase (1 mg), DPN (0·001M), $MgSO_4$ (0·001M), and AA (0·0005M). Final volume 2 ml.

† Complete reaction mixture contains enzyme (0·6 ml), sucrose (0·4M), phosphate (0·02M), glucose (0·02M), hexokinase (0·2 mg), glucose-6-phosphate dehydrogenase (1 mg), TPN (0·001M), $MgSO_4$ (0·001M), and adenosine triphosphate (0·001M). Final volume 2 ml.

The cofactor requirements are similar to those needed for comparable preparations from both animal and plant tissues, although the reason for the additional stimulation by the coenzyme extract is obscure. It is not due to any lack of DPN (Table 2), nor in etiolated pea stems can it be attributed to the lack of TPN (Table 4). Further, the stimulation is probably not due to coenzyme *A* present in the Coe, since a stimulation of approximately the same magnitude occurs when succinate is the substrate. These considerations, together with the fact that the AA requirement shows that the oxidative rate is limited by the rate of phosphorylation (cf. Laties 1953*b*) suggest that the site of action of the Coe effect is somewhere along the pathway of phosphorylation.

Hitherto, it has not been possible to demonstrate phosphorus : oxygen (P : O) ratios in particulate preparations from plants comparable with those observed in preparations from animal tissues (Biale 1953; Bonner and Millerd 1953). Although a P : O ratio greater than unity was not obtained for the oxidation of reduced DPN and TPN, the P : O ratios obtained with succinate and α-ketoglutarate are comparable with those obtained with isolated animal mitochondria, and it would appear justified to assume a phosphorylation quotient of

TABLE 6

OXIDATION OF SUBSTRATES OF THE TRICARBOXYLIC ACID CYCLE BY PARTICLES FROM VARIOUS TISSUES OF THE PEA PLANT

Composition of the reaction mixture as in Table 1. Substrate concentration 0·02M except DL-malate (0·0015M)

Material	Oxygen Uptake (μl O_2/mg N/30 min)						
	No Substrate	Malate	Malate + Pyruvate	α-Keto-glutarate	*iso*Citrate	Succinate	Glutamate
Green leaf (i)	16	—	—	182	116	232	91
(ii)	—	25	256	—	—	—	—
Green stem	55	72	159	342	213	562	256
Root (i)	17	—	—	330	73	164	78
(ii)	—	45	193	—	—	—	—
Etiolated leaf	7	100	286	350	306	598	229
Etiolated stem	0	69	380	405	254	747	384
Germinated seed	50	59	404	277	172	372	188
Fresh pea seed	17	22	142	110	86	210	127
Hull	48	104	260	240	—	168	156

2 for the reaction succinate ⟶ fumarate, and of 4 for the reaction α-ketoglutarate ⟶ succinate (cf. Krebs 1954). It is interesting to note that light has no significant effect on the rate of oxidation or phosphorylation in preparations containing both mitochondria and whole chloroplasts (Table 3).

The oxidation of butyrate merits some consideration. Previously Newcomb and Stumpf (1952) demonstrated the oxidation of palmitate by cell free preparations of peanut cotyledons. In particular, palmitate, but not any of the

TABLE 7

THE OXIDATION OF BUTYRATE BY PARTICLES FROM VARIOUS TISSUES OF THE PEA PLANT

The reaction mixture except for substrates is as in Table 1

Material	Oxygen Uptake (μl O_2/mg N/30 min)	
	Malate	Malate + Butyrate
Etiolated stem*	37	90
Etiolated first leaf*	57	162
Root*	78	129
Germinated seed†	59	180
Fresh pea seed†	28	97

* DL-Malate (0·003M), butyrate (0·02M).

† DL-Malate (0·0015M) and butyrate (0·05M).

lower fatty acids, was oxidized by a cell fraction containing particles of microsomal size, but no oxidation of fatty acids occurred with the mitochondrial fraction. While it is true that the particulate fraction, prepared as already described, would probably contain the larger microsomes, as well as the still larger mitochondria, the initial high rate of butyrate oxidation indicates that this oxidation is taking place on the same particles which bring about the oxidation of the tricarboxylic acid cycle substrates. Both processes are in some way related since small amounts of malate appreciably increase the rate of butyrate oxidation, and it hence appears fairly certain that enzymes contained in the mitochondria are essential for this oxidation, although the possible participation of microsomal particles cannot be discounted. Further work is proceeding towards a more positive identification of the site of butyrate oxidation.

V. Conclusion

The above results indicate that the enzymes of the tricarboxylic acid cycle are universally distributed in the cells of the pea plant and that these enzymes are located as a complete integrated system on sub-cellular particles known as mitochondria. The high oxidative activities obtained, together with the demonstration of P : O ratios comparable with those obtained with animal mitochondria during the oxidation of α-ketoglutarate and succinate, lead to the conclusion that the tricarboxylic acid cycle-cytochrome system is the major respiratory mechanism operating in the cells of either etiolated or actively photosynthesizing pea plants.

VI. Acknowledgments

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VII. References

Allen, R. J. L. (1940).—*Biochem. J.* **34**: 858.
Arnon, D. I., and Whatley, F. R. (1949).—*Arch. Biochem.* **23**: 141.
Biale, J. B. (1953).—Abstr. Amer. Soc. Plant Physiol. Sept. p. 21.
Berger, L., Slein, M. W., Colowick, S. P., and Cori, C. F. (1946).—*J. Gen. Physiol.* **29**: 279.
Bonner, J., and Millerd, A. (1953).—*Arch. Biochem. Biophys.* **42**: 135.
Brummond, D. O. (1952).—M.S. Thesis, University of Wisconsin.
Brummond, D. O. (1953).—Abstr. Amer. Soc. Plant Physiol. Sept., p. 38.
Davies, D. D. (1953).—*J. Exp. Bot.* **4**: 173.
Hackett, D. P., and Simon, E. W. (1954).—*Nature* **173**: 162.
Krebs, H. A. (1954).—In "Chemical Pathways of Metabolism." Vol. 1. p. 109. (Ed. D. M. Greenberg.) (Academic Press: New York.)
Krebs, H. A., and Eggleston, L. V. (1944).—*Biochem. J.* **38**: 426.
Le Page, G. A., and Mueller, G. C. (1949).—*J. Biol. Chem.* **180**: 975.
Laties, G. G. (1953*a*).—*Plant Physiol.* **28**: 557.
Laties, G. G. (1953*b*).—*Physiol. Plant.* **6**: 215.
Millerd, A., Bonner, J., and Biale, J. B. (1953).—*Plant Physiol.* **28**: 521.

MILLERD, A., BONNER, J., AXELROD, B., and BANDURSKI, R. (1951).—*Proc. Nat. Acad. Sci. Wash.* **37**: 855.

NEWCOMB, E. H., and STUMPF, P. K. (1952).—In "Phosphorus Metabolism." Vol. 2. p. 291. (Johns Hopkins Press: Baltimore.)

NEWCOMER, E. H. (1951).—*Bot. Rev.* **6**: 53.

PEARSON, J. A., and ROBERTSON, R. N. (1954).—*Aust. J. Biol. Sci.* **7**: 1.

PRICE, C. A., and THIMANN, K. V. (1954).—*Plant Physiol.* **29**: 113.

RACKER, E. (1950).—*J. Biol. Chem.* **184**: 313.

SHARPENSTEEN, H. H., and CONN, E. E. (1954).—Abstr. Amer. Soc. Plant Physiol. Sept. p. 37.

SLATER, E. C., and CLELAND, K. W. (1953).—*Biochem. J.* **55**: 566.