SUMMARY

1. Protein turnover was studied in growing and non-growing cell suspensions of *Bacillus cereus*.

2. The average rate of protein breakdown in growing cells was about 1.4 %/hr. In non-growing cells the rate of both protein synthesis and protein breakdown was about 7 %/hr.

3. Chloramphenicol inhibited both synthesis and degradation of protein in non-growing *B. cereus* cells.

4. Very little cell lysis occurred and it therefore appears that protein turnover is very largely an intracellular process.

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Mitochondrial Preparations from the Leaves of Tobacco (*Nicotiana tabacum*)

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There is abundant evidence that the tissues of higher plants contain mitochondria and that, when these are extracted by methods similar to those used for animal mitochondria, they are associated with the oxidation and phosphorylation reactions of the tricarboxylic acid cycle (Hackett, 1955; Laties, 1957). Most of the studies on this isolation have, however, been concerned with etiolated or non-pigmented tissues. Very few have been done on chlorophyllous tissue, as the presence of plastids and the vacuolar contents make extraction and purification difficult.

Because of these difficulties some workers have concluded that in green leaves many of the reactions of the tricarboxylic acid cycle are not associated with mitochondria (Brummond & Burris, 1954), or that some of them are located in the plastids (Ohmura, 1955; Sissakian, 1955). But recently mitochondria similar to those from etiolated tissues have been prepared from the leaves of peas (Smillie, 1956a, b; Sissakian, Bekima &

Mosolova, 1957) and other plants (James & Das, 1957). Good evidence is presented, especially in the last of these papers, that the oxidations of the tricarboxylic acid cycle are located exclusively in these mitochondria and not in the chloroplasts.

The present work describes the preparation and some of the properties of an extract of tobacco leaves which is sedimented between 1000 and 10 000 g and which brings about many of the oxidations of the tricarboxylic acid cycle. Although no cytological observations on it are presented, it seems likely that it is a preparation, in a damaged and impure form, of leaf mitochondria. It is therefore referred to as such throughout this paper.

EXPERIMENTAL

Plants. Seeds of *Nicotiana tabacum* (White Burley strain) were germinated in a heated greenhouse in John Innes seed compost, and transplanted as soon as possible into John Innes potting compost. The plants were allowed to grow for at least another 5 weeks before they were used.

Mitochondrial preparations. In a typical experiment all the green leaves were removed from two or three plants and cleaned either by careful dusting with filter paper or by immersion in water followed by blotting with filter paper. A comparison of the two methods showed no difference, so the former, quicker, one was adopted. The midribs were then cut out and the leaf material was weighed.

About 25–30 g. of this tissue was cut into strips and ground in a chilled mortar containing 65 ml. of extraction medium and 15 g. of acid-washed sand. When more leaf tissue was used, the amount of medium was increased proportionally. The resulting suspension was passed, with gentle squeezing if necessary, through two layers of butter muslin, and the sand and fibre residue discarded. The filtrate was then centrifuged for 7 min. at a centrifugal force of approx. 1000 *g* at the centre of the centrifuge tube, to give a sediment containing starch and chlorophyllous material but very little succinoxidase. This is referred to as the 'chloroplast fraction'. The supernatant was further centrifuged for 30 min. at a force of approx. 10 000 *g* to give a green 'mitochondrial' pellet and a relatively clear supernatant solution.

The mitochondrial pellet was suspended evenly in about 40 ml. of cold washing medium, with the aid of a large test tube and a loose-fitting glass plunger, and sedimented by centrifuging at 10 000 *g* for 30 min. For use in enzymic tests it was suspended evenly in 0.25 M-sucrose and 0.4 or 0.5 ml. samples, containing about 1 mg. of protein nitrogen, were pipetted into appropriate tubes.

Oxygen uptake. Oxygen uptake was measured in the conventional Warburg apparatus by the techniques recommended by Umbreit, Burris & Stauffer (1949). The flasks, containing the complete reaction medium in their main compartments and 0.2 ml. of 10% KOH and folded pieces of filter paper in their centre wells, were cooled in ice before the addition of plant extract. After transfer to the water bath at 30°, they were shaken at a rate of 120 strokes/min. and allowed to equilibrate for 10 min. before the taps were closed.

Oxygen uptake was proportional to the amount of enzyme added up to 4 mg. of protein N. It was generally measured for 90 min., but was expressed as μ l. of oxygen/mg. of protein N/30 min. (q_{O_2}).

The medium in which the mitochondrial preparations were incubated had the composition: substrate acid 25 mM; sucrose 0.2 M; phosphate buffer, 66 mM, pH 7.3; magnesium sulphate 6.6 mM; adenosine triphosphate (ATP) 1.3 mM; cytochrome *c* 0.102 mM. The final volume was 1.5 ml. and the pH 7.2–7.3. Any deviations from these conditions are mentioned in the appropriate places.

Chemical estimations. Citric acid was estimated by a combination of the methods of Schneider, Striebig & Hogeboom (1956) and McArdle (1955). The citrate was converted into pentabromoacetone with KBr and KMnO₄ by the method of Schneider *et al.* (1956), but this was extracted into light petroleum (b.p. 80–100°) and a colour developed with the thiourea-borax-sodium sulphide solution of McArdle. The colour was read at 490 $m\mu$, its wavelength of maximum absorption, in the Unicam SP. 500 spectrophotometer. This method has the advantages that there is no interference from the sucrose in the plant extracts as there is in the method of McArdle, and it avoids the use of pyridine as an extractant.

Protein nitrogen was determined by a micro-Kjeldahl method. The protein samples (0.1–0.2 ml.) were first pre-

cipitated with 2 ml. of 15% (w/v) trichloroacetic acid, centrifuged down at 1500 *g*, washed with 7.5% (w/v) trichloroacetic acid and then dissolved in the minimal amount of N-NaOH soln. These solutions were then digested with 1 ml. of conc. H₂SO₄ and approx. 0.2 g. of a catalyst (composition: K₂SO₄ 90 g.; CuSO₄·5H₂O 10 g.; SeO₂ 0.1 g.). The ammonia was distilled into boric acid solution (2%), and measured by titration with HCl.

Chlorophyll was estimated, after extraction in aq. 80% (v/v) acetone, by measuring the absorption of the filtered solution at 663 and 645 $m\mu$ (Arnon, 1949).

Chromatography of organic acids. Organic acids were extracted with ether from the acidified (pH 2) mitochondrial suspensions for 50 hr. and, after the ether had been evaporated, were dissolved in 0.5 ml. of water. Varying amounts (12, 25 and 40 μ l.) of these solutions were put on to sheets of Whatman no. 1 filter paper, in 3 μ l. spots, and the chromatograms developed in an ethanol-water-aq. NH₃ soln. solvent (Jones, Dowling & Skraba, 1953). After 24 hr. the papers were removed, dried, sprayed with ammoniacal silver nitrate and heated for 7 min. in an oven at 95° (Brown, 1951). The appearances of the acid spots under u.v. light are characteristic, and are a useful aid to *R_f* values in identification. Thus citrate, isocitrate and malate give intense-yellow fluorescent spots, the citrate and isocitrate ones being rather more orange than the other. Succinate, fumarate and *cis*-aconitate give blue spots, and α -oxoglutarate a dull red one. Oxaloacetate and pyruvate could not be detected.

Chemicals. Cytochrome *c* was prepared from horse heart by the method of Keilin and Hartree as modified by Umbreit *et al.* (1949). Pyruvic acid was prepared from tartaric acid according to Howard & Fraser (1948) and converted into the sodium salt by the method of Price & Levintow (1952). A 10% coenzyme A concentrate was generously given by Dr E. M. Crook, and a crude yeast hexokinase preparation by the Sigma Chemical Co.

All other chemicals were obtained commercially, and were AnalR as far as possible. The acid-washed sand (Hopkin and Williams Ltd.) was further washed with 0.05 M-ethylenediaminetetra-acetic acid (EDTA) and water and then air-dried before it was used. High molecular-weight dextrans were obtained as Intradex (British Drug Houses Ltd.), a 6% solution with 5% of added glucose. Carbowax 4000, a preparation of high molecular-weight polyethylene glycols (General Metallurgical and Chemical Ltd.), was purified by precipitation from acetone (McClendon, 1954).

Phosphate buffers were prepared from M-NaH₂PO₄ by adding conc. NaOH soln., and diluting them to the required molarity. Tris buffers were similarly made from 2-amino-2-hydroxymethylpropane-1,3-diol and HCl. The solutions were finally adjusted to the required pH values after dilution. Measurements were made with a glass electrode that had been calibrated with a saturated solution of potassium hydrogen tartrate and commercial buffers (Burroughs Wellcome and Co.).

RESULTS

Effect of the composition of the extraction medium on succinoxidase activity

The composition of the extraction medium in which the leaves are ground drastically affects the activity of the mitochondrial preparations. The

most active succinoxidase preparations, with low endogenous activity, were made by grinding the leaves in a heavily buffered modification of the sucrose medium that was introduced so successfully for animal mitochondria (Hogeboom, Schneider & Pallade, 1948). Its composition was sucrose 0.4M, 2-amino-2-hydroxymethylpropane-1:3-diol (tris) 0.2M, potassium phosphate 0.01M, EDTA 5 mM, and sodium citrate 0.02M at a final pH of 7.8-7.9. After their initial sedimentation at 10 000 g, preparations made in this medium were washed in a medium containing the same substances at half the above concentrations, but only 5 mM-citrate, at a final pH of 7.2-7.3.

The effect on succinoxidase of omitting each of the components in turn from the extraction and washing media is shown in Table 1. In these experiments the leaves with midribs removed were cut longitudinally into two roughly equal parts, and these parts were assigned to different batches. One of these batches of half-leaves was then disintegrated in the standard medium described above and the other in the modified medium. This procedure minimizes differences between the two preparations due to differences in starting material. When the two preparations were made in samples of the same medium, differences in their activity were small (Table 1, Expt. 9). Thus differences between the two preparations due to factors other than the differences in medium can be neglected.

Omission of any one of the constituents, with the exception of phosphate, leads to a decrease in the succinoxidase activity of the preparation. With omission of sucrose (Table 1, Expts. 1 and 2), the 30-40% decrease may not be a destruction of activity but rather a redistribution. Without sucrose the medium is less dense and viscous, and

a larger proportion of the particles bearing succinoxidase may be sedimented by the initial centrifuging at 1000 g. Losses due to omission of the other constituents are probably due to destruction of the enzyme system.

The inclusion of phosphate in the medium had no beneficial effect on the succinoxidase (Table 1, Expt. 8), even when its concentration was increased tenfold (Table 1, Expt. 10). It was retained in the medium at a low concentration, however, as it is known to affect beneficially plant-mitochondrial activities other than succinoxidase (Millerd, Bonner, Axelrod & Bandurski, 1951), and to have an effect in the extraction medium besides that due to its buffering properties (Millerd, 1953).

The omission of EDTA from the extraction medium results in a 10-30% decrease in the succinoxidase (Table 1, Expts. 4 and 5). This protective effect of EDTA on oxidations carried out by plant-mitochondrial preparations is well known (Smillie, 1955). It is explained as due to the chelation of Ca^{2+} ions from the disintegrated leaves (Lieberman & Biale, 1955), and is more marked in preparations made from tissues with highly vacuolated cells (Lund, Vatter & Hanson, 1958).

The pH of the supernatants left after the chloroplast and mitochondrial fractions had been centrifuged out of the leaf extracts were generally between 7.0 and 7.85. Those of the experiments described in Table 1 were all between 7.4 and 7.7, and the two supernatants obtained in each experiment were within 0.1 pH unit of each other, except when tris, the major buffering component of the medium, was omitted (Table 1, Expt. 3). The pH of the supernatant then dropped to 6.3, about 1.2 units below the value of the control preparation, and there was a 60% loss in the succinoxidase

Table 1. *Effect of composition of the extraction medium on succinoxidase activity*

Batches of leaves (35-50 g.) were cleaned, removed from the midrib and divided into two equal portions of half-leaves. Mitochondria were prepared from one portion with the standard extraction and washing media, whose compositions were sucrose 0.4M, tris 0.2M, EDTA 5 mM, phosphate 0.01M, citrate 0.02M, pH 7.9, and sucrose 0.2M, tris 0.1M, EDTA 2.5 mM, phosphate 5 mM, citrate 5 mM, pH 7.3. The media used for the other portions were modified as described below. Succinoxidase activity was measured as described in the Experimental section.

Expt. no.	Standard medium		Modification	Modified medium	
	Endogenous q_{O_2}	Succinoxidase q_{O_2}		Endogenous q_{O_2}	Succinoxidase q_{O_2}
1	13.4	43	Sucrose omitted	13.2	33
2	10	45	Sucrose omitted	9	29
3	6	40	Tris omitted	11	24
4	7	43	EDTA omitted	5.5	38
5	12	48	EDTA omitted	13	37
6	10.6	60	Citrate omitted	21	31
7	14	70	Citrate omitted	11	32
8	13	125	Phosphate omitted	14	132
9	—	—	Phosphate omitted	3.8	37.5
	—	—	Phosphate omitted	4.8	35
10	—	—	Phosphate omitted	3.8	50
	—	—	10 times as much phosphate	3.1	45

activity. This loss of activity when the pH of the extract is below 6.8 is also illustrated in Table 2. However, as this table also shows, wide variations in the final pH have comparatively little quantitative effect on the succinoxidase activity.

Lieberman (1958) has recently reported that an extractant with a high buffering capacity (0.2M-tris, pH 9.2) is necessary to obtain a sedimentable cytochrome oxidase from apples. With this enzyme, as with the present succinoxidase of tobacco leaves, no activity could be obtained by buffering the extraction medium at pH 7.0 with phosphate.

The largest decreases in succinoxidase (60–80 %) recorded in Table 1 were caused by the omission of citrate from the extraction and washing media (Expts. 6 and 7). But whereas this protective effect of citrate has been observed more than six times, an occasional very active preparation was made in the absence of citrate. One such preparation had an endogenous q_{O_2} of 9, which was increased to 45 by the presence of succinate. This inconsistency, which may be due to differences in the physiological state of the leaves, is similar to that found with spinach leaves (Ohmura, 1955). Sedimentable extracts prepared from these leaves in the absence of citrate were 'often' unable to oxidize added organic acids.

It is not at all certain what the mechanism of this protective effect of citrate is. It may involve the removal of succinoxidase inhibitors from the preparation, since citrate added to a preparation after it had been squeezed through muslin had a beneficial effect. But as these preparations catalyze some of the reactions of the tricarboxylic acid cycle, it could be interpreted as due to the formation of succinate and the consequential stabilization of the succinoxidase by its substrate. Consistent with this view is the fact that succinate itself will replace citrate in the extraction medium (Table 3). Fumarate will not replace citrate, but these tobacco-leaf extracts, in common with some other plant-mitochondrial preparations (Switzer & Smith, 1957), have little or no oxidative activity for fumarate.

Amount of extraction medium

The amount of extraction medium in which the leaf tissue was ground was initially chosen for convenience of grinding. That it may not be the optimum amount for the recovery of succinoxidase is indicated by an experiment in which 44 g. of leaves was divided into two equal lots of half-leaves, and one lot ground, with 15 g. of sand, in 50 ml. of extraction medium and the other in 90 ml. Succinoxidase prepared from the first lot had q_{O_2} 29 and an endogenous q_{O_2} of 9.2. That from the second lot had a higher q_{O_2} (46) and a lower endogenous q_{O_2} (4). This effect may be explained as

the dilution of some inhibitory material by the extraction medium.

In many of the more recent experiments the amount of extraction medium used for 25–30 g. of leaves has been increased to 85 ml.

Other extraction media

The inclusion of ascorbate (0.01M) in the extraction medium had no measurable effect on the succinoxidase activity. Nor had the inclusion of fluoride (0.01M), which protects the succinoxidase of young barley plants by inhibiting an interfering lecithinase (Goodwin & Waygood, 1954).

No succinoxidase could be detected in preparations made by grinding tobacco leaves in a variety of media containing Carbowax 4000 (20 %, w/v), although these polyethylene glycols have been

Table 2. *Effect of pH of extraction medium on succinoxidase activity*

In each experiment the leaves were divided into two equal lots of half-leaves, and both lots disintegrated in the standard extraction medium whose pH had been adjusted to different values. Succinoxidase was prepared as described in the Experimental section, and the pH values of the supernatant solutions were measured.

Expt. no.	pH of supernatant	Endogenous q_{O_2}	Succinoxidase q_{O_2}
1	7.55	12	55
	8.4	15	61
2	7.9	9	31
	8.6	11	30
3	6.8	17	42
	6.0	20	30

Table 3. *Replacement of citrate in the extraction medium*

In each experiment the leaves were divided into two equal lots of half-leaves, and succinoxidase was prepared from each lot in the media indicated below. The media used were: the standard one containing citrate (0.02M-citrate in the extraction medium and 5 mM in the washing medium); one in which the citrate was replaced by fumarate (0.02M and 5 mM); one in which it was replaced by succinate (10 and 0.2 mM); and one in which it was omitted and not replaced. Succinate was used at lower concentrations to prevent its being carried over into the succinoxidase estimation.

Expt. no.	Medium	Endogenous q_{O_2}	Succinoxidase q_{O_2}
1	Citrate present	8.9	42
	Succinate present	6	38.5
2	Neither present	6	16
	Succinate present	6	34
3	Citrate present	12.5	75
	Fumarate present	12	30
4	Neither present	12	23
	Fumarate present	15.5	28

reported to have a beneficial effect on isolated tobacco chloroplasts (McClendon & Blinks, 1952; McClendon, 1954). Similarly, there was little or no succinoxidase in extracts prepared in a medium similar to that described by Birbeck & Reid (1956), and containing raffinose, dextran and heparin. However, with this medium, the addition of citrate had a beneficial effect (Fig. 1).

Distribution of succinoxidase

The chloroplast and mitochondrial fractions obtained by the usual procedure each contained about 15 % of the total protein nitrogen of the extracts. The bulk of the recovered succinoxidase was located in the less dense mitochondrial fraction. In order to check these distributions, a number of fractionations were carried out more thoroughly and each sedimented fraction was washed twice in the usual washing medium. The pooled washings of

Fig. 1. Effect on succinoxidase of including citrate in the extraction medium. Tobacco leaves (40 g.) were cleaned and midribs were removed and then divided into two equal lots of half-leaves. Succinoxidase was prepared from one lot in an extraction medium [raffinose hydrate (0.23 M), Intradex (6%, v/v), tris (0.15 M), heparin (0.035%, w/v), EDTA (2 mM), adenosine monophosphate (AMP, 0.6 mM), final pH 7.8] and washed in the same medium without AMP, at pH 7.4. Succinoxidase was prepared from the second lot of half-leaves in the same medium, but in the presence of citrate (15.4 mM). Samples of both preparations, containing 1.2 (----) and 1.5 (—) mg. of protein N respectively, were used in measuring endogenous oxygen uptake (○) and succinoxidase activity (●).

Table 4. *Distribution of succinoxidase among fractions of tobacco leaves*

Fraction	Protein N		(mg.)	Chlorophyll		Oxygen uptake			Total succinoxidase in the fraction (%)
	(mg.)	(% of total)		(mg./mg. of protein N)	Endogenous q_{O_2}	Succinoxidase q_{O_2}	Total		
								(% of total)	
Initial extract	82	100	23.5	100	0.29	9.0	17	100	
Chloroplast	8.5	10.4	8.8	38	1.04	7.5	8.9	2	
Mitochondrial	12.2	15	8.6	35	0.73	7.6	32	50	
Supernatant	58	71	0.9	4	0.015	4	5.0	9	

The leaves (28 g.) were disrupted in the usual extraction medium (70 ml.) and fractions sedimented at 1000 and 10 000 g. Each sediment was washed twice in the washing medium, and the pooled washings were added to the supernatant extract for the next centrifuging. Chlorophyll, succinoxidase and protein N were measured on samples of each fraction. Endogenous oxygen uptake was subtracted from uptake in the presence of exogenous substrate in calculating the total succinoxidase activity.

Table 5. *Effect of the state of leaves on succinoxidase activity*

In each experiment succinoxidase was prepared from 16 g. of sticky leaves from plants over 4 months old, and also from 16 g. of non-sticky leaves from younger plants. The extraction medium and method of preparing and measuring succinoxidase activities were the standard ones described in the text.

Expt. no.	Succinoxidase from sticky leaves		Succinoxidase from non-sticky leaves	
	Endogenous q_{O_2}	Exogenous q_{O_2}	Endogenous q_{O_2}	Exogenous q_{O_2}
1	18	22	6.4	52
2	18	31	15	75

Fig. 2. Effect of age of leaf on succinoxidase activity. The green leaves were taken from five tobacco plants and separated into young ones, less than 20 cm. long, and older ones. Succinoxidase was prepared from 20 g. of young leaves (—) and 20 g. of old leaves (----) with the media and methods described in the text. Samples of both preparations, containing 1.8 and 1.6 mg. of protein N respectively, were taken for measuring endogenous oxygen uptake (○) and uptake in the presence of succinate (●).

the chloroplast fraction were added to the supernatant from which the mitochondrial fraction was to be sedimented. Table 4 shows the distribution of protein nitrogen, chlorophyll and succinoxidase in one of these experiments.

The figures for the total succinoxidase activity in the last column are calculated from the differences between the endogenous and exogenous activities of each fraction. Since the endogenous activities of the initial extract and the supernatant fractions are high, no doubt owing to the presence of oxidizable organic acids including citric acid from the extraction medium, these figures are only approximate. But they suggest that the bulk of the recoverable succinoxidase is in the mitochondrial

fraction. This fraction is the only one in which the succinoxidase is enriched, on a protein nitrogen basis, relative to the initial extract.

Chlorophyll is almost equally divided between the chloroplast and the mitochondrial fractions; in both it is more concentrated, relative to protein nitrogen, than in the initial extract. The chlorophyll:protein nitrogen ratio in the chloroplast fraction is similar to that reported by other workers for tobacco-leaf chloroplasts (Jagendorf & Wildman, 1954; Jagendorf, 1955). Microscopic examination showed that the mitochondrial extract, unlike the other, contained very few apparently intact chloroplasts. But since the chlorophyll:protein nitrogen ratio of chloroplast fragments is the same as that for intact chloroplasts (Jagendorf & Wildman, 1954), about 70 % of the protein nitrogen of the mitochondrial fraction is probably derived from chloroplasts. The chloroplasts therefore account for about 20 % of the protein of the leaf extracts. Only a third of the protein of the mitochondrial fraction, corresponding to 5 % of the protein in the leaf extract, is derived from mitochondria and cell components other than chloroplasts.

Effect of the physiological state of the plant on succinoxidase

Considerable variation in the activity of preparations made by the same method was encountered. This variation, of which something can be seen in Table 1 (even though results obtained with preparations of low activity have been omitted), is probably due to differences in the physiological state of the leaves. As a rough generalization, preparations made from older plants (3–4 months), whose leaves were covered with a sticky exudate, were poor (Table 5), and washing these leaves with water to remove the exudate had no beneficial effect. But this is complicated by the fact that the younger, smaller, leaves of a plant gave more active preparations than the older leaves (Fig. 2). Some quite active preparations have been made from the smaller leaves of very old and sticky plants. The leaves from which a high succinoxidase would be expected seem to correspond to those from which largest

Table 6. *Omission of substances from the succinoxidase-assay system*

Succinoxidase was prepared from tobacco leaves in the usual way. Its activity was measured in a medium whose final composition was: succinic acid 25 mM; sucrose 0.2M; phosphate buffer, pH 7.3, 66 mM; magnesium sulphate 6.6 mM; ATP 1.3 mM; cytochrome *c* 12 μ M; and also in a similar medium with the modifications listed below.

Activity is expressed as a percentage of that in the modified medium after the subtraction of the endogenous oxygen uptakes.

Expt. no.	Component altered	Concn. (M)	Succinoxidase activity (%)
1	Magnesium sulphate	0	100
		0.0066	100
2	Magnesium sulphate	0	100
		0.0066	131
3	Cytochrome <i>c</i>	0	100
		2.4×10^{-8}	100
		2.4×10^{-7}	150
		2.4×10^{-6}	280
		2.4×10^{-5}	280
4	Sucrose	0.062	100
		0.187	146
		0.376	152

Table 7. *Variation of succinoxidase activity with succinate concentration*

Tobacco leaves (25 g.) were ground in 65 ml. of the usual extraction medium, and the enzyme was prepared as described. Succinoxidase activity was measured on samples containing 0.63 mg. of protein N, in a medium of composition: sucrose 0.2M; phosphate buffer, pH 7.3, 66 mM; magnesium sulphate 6.6 mM; ATP 1.3 mM; cytochrome *c* 12 μ M; succinate as stated below. Endogenous oxygen uptake is subtracted before the figures in the last column are derived.

Succinate concn. (mM)	Oxygen uptake (q_{O_2})	Relative succinoxidase activity (%)
150.0	39	58
50.0	60	100
25.0	53	96
12.5	44	69
6.2	27	33
0	11	—

amounts of a slowly sedimenting nucleoprotein have been isolated (Pirie, 1950).

Oxidative activity of preparations made from the leaves of pea plants have been reported to vary with the conditions under which plants were grown. Smillie (1956*a*) noted an adverse effect of high temperatures and humidity, and Sissakian *et al.* (1957) showed a similar effect of optimum illumination coupled with temperatures above 20°.

Factors affecting the estimation of succinoxidase

The composition of the medium in which succinoxidase was measured is given in the Experimental section. It was based on media used for plant tissues by other workers. The importance of each of its components was measured by omitting them singly, when it was found that only the ATP and

phosphate were consistently without effect on the oxygen uptake. Other derivatives of adenylic acid, adenosine di- and mono-phosphate, were also ineffective. Nevertheless, both ATP and phosphate were always added in view of possible requirements for the oxidation of other substrates and phosphorylative reactions.

The effect of omitting other components from the media are collected in Table 6. The small effect of magnesium, quite unlike the larger one of cytochrome *c*, was rather variable. Cytochrome *c* was active in very small amounts, 2.4 μ M being sufficient for maximum activity. Sucrose was never completely omitted from the medium, since the enzyme was suspended in 0.25M-sucrose for its distribution to the Warburg flasks. However, additional sucrose is necessary for maximum activity (Table 6). No inhibition of the oxidation of succinate was observed with higher concentrations of sucrose (0.38M), as occurs with some animal preparations (Johnson & Lardy, 1958).

The optimum concentration of substrate succinate is near 25 mM, the concentration at which it was used. At higher concentrations there was a decrease in activity (Table 7). A substrate inhibition at a similar concentration has been reported for at least one other plant succinoxidase (Damodaran & Venkatesan, 1941).

The pH optimum of the succinoxidase, measured in solutions buffered with a mixture of 12.5 mM-tris-12.5 mM-phosphate, was between 6.8 and 7.2. The activity falls away steeply in the alkaline region so that it has half gone at pH 8. It falls away less steeply in acid pH values. The pH region of optimum activity is therefore similar to those for succinoxidases of bean seedlings (Damodaran & Venkatesan, 1941) and potato tubers (Millard, 1951).

Substances which have stimulated oxidations of organic acids catalysed by various plant extracts include glucose and hexokinase (Smillie, 1956*a*) and glutamic acid. These had no effect on the succinoxidase of tobacco leaves.

Small amounts (0.3 ml.) of the supernatant fraction of tobacco leaves had no effect on the oxidation of succinic acid by the mitochondrial preparations, but they stimulated two- or three-fold the endogenous oxygen uptake of the preparations. This endogenous stimulation was not destroyed by boiling the supernatant for a few minutes. It was probably due to the oxidation of the organic acids in the supernatant fraction.

Oxidation of substrates of the tricarboxylic acid cycle

Most of the substrates of the tricarboxylic acid cycle were oxidized, to various degrees, by the tobacco-leaf mitochondrial preparations. Although the relative activity towards different substrates varies somewhat with the preparation, the values recorded in Table 8 are typical. Succinate was the most actively oxidized substance, and fumarate and isocitrate were oxidized very slowly.

Table 8 also shows how the addition of coenzyme A, thiamine pyrophosphate and diphosphopyridine dinucleotide affected these oxidations. All, except that of succinate, were stimulated two- to four-fold.

Attempts to increase the low rate of fumarate oxidation were unsuccessful. They included alteration to the concentration of substrate and sucrose and the pH of the medium. The low oxidation rate of fumarate has already been remarked upon (Switzer & Smith, 1957).

Oxidation of mixtures of substrates

The presence of other oxidizable substrates during the oxidation of succinate did not increase the amount of oxygen absorbed. Often it depressed it slightly. Thus the rate of oxidation of mixtures of succinate (25 mM) and citrate (25 mM) was 90 % of the rate of oxidation of succinate alone. Similarly the oxidation of malate and succinate was 94 % of the succinate value. This effect suggests that the oxidation of these substances have common steps, and that these are saturated during the oxidation of succinate.

The increase in the oxygen uptake of tobacco-leaf extracts caused by the presence of pyruvate (25 mM) was a mere 5–8 % of that due to succinate. The addition of the cofactors used in Table 8, and small 'sparker' quantities (1.2 mM) of malate, succinate or oxaloacetate did not alter this amount. Appreciable oxidation of pyruvate, 'sparked' by one of the other substrates of the tricarboxylic acid cycle, was only obtained when the leaves were infiltrated with dilute ammonia solution, *in vacuo*, before grinding (Fig. 3).

Table 8. *Oxidation of intermediates of the tricarboxylic acid cycle by mitochondrial extracts of Nicotiana tabacum*

The mitochondrial preparations were made and tested for ability to oxidize these intermediates as described in the Experimental section. Oxidation was also measured in the presence of the additional cofactors thiamine pyrophosphate (0.13 mM), diphosphopyridine dinucleotide (0.13 mM) and coenzyme A (13 μ M). Endogenous oxygen uptake, which was unaffected by the cofactors, was subtracted from the uptakes obtained with the substrates. The results are expressed as a percentage of that due to succinate in the absence of cofactors.

Substrate	Oxygen uptake (% of succinate activity)	Oxygen uptake in the presence of cofactors (% of succinate activity)
Succinate	100	109
Fumarate	3	19
Malate	37	100
Oxaloacetate	0	0
Citrate	31	64
<i>cis</i> -Aconitate	30	61
<i>iso</i> Citrate	4	13
Oxalosuccinate	10	42
α -Oxoglutarate	25	90

Fig. 3. Oxidation of pyruvate. Tobacco leaves (25 g.) were cleaned, shredded and immersed, in a desiccator, in 1 l. of cold aq. NH_3 soln. that had been adjusted to pH 8 with HCl. The desiccator was then evacuated for 30 min. at the water pump. The leaves were washed in water, blotted dry and a mitochondrial preparation was made from them. Samples of this preparation containing 0.85 mg. of protein N were taken for the measurement of endogenous oxygen uptake (○), as well as uptake in the presence of 25 mM-succinate (■), 25 mM-pyruvate (□), 1.2 mM-malate (△) and 25 mM-pyruvate plus 1.2 mM-malate (●).

Products of the oxidations

In order to identify the products formed by the oxidation of the substrates, the reactions were stopped after 90–120 min. by adjusting to pH 2 with HCl and heating for 1 min. at 100°. Denatured protein was then removed by centrifuging and an ether extract of the solution examined by paper chromatography. Controls were usually done on mitochondrial preparations that had been incubated without substrate, and a mixture of enzyme and substrate that had not been incubated. By this means it was shown that the oxidation of succinate gave rise to malate, and the oxidation of citrate gave rise to succinate and malate.

When α -oxo acids were substrates, the boiling was omitted as it caused some decomposition. Even without it a slight decomposition of α -oxoglutarate to succinate was observed in the unincubated controls. It was, however, only a small fraction of the succinate formed on incubation. Malate and a trace of citrate were also formed on incubation. The products identified after the oxidation of oxaloacetate were malate and citrate.

These experiments are consistent with the view that the substrates were being oxidized by one or more of the reactions of the tricarboxylic acid cycle. The formation of malate and citrate from oxaloacetate was surprising in view of the lack of oxygen uptake in the presence of oxaloacetate (Table 8). It may, however, be due to a dismutation of the type

This would involve no oxygen uptake and has been observed with the mitochondria of other plants (Avron & Biale, 1957).

Table 9. *Effect of malonate on oxidations by mitochondrial preparations*

Mitochondrial preparations were made from tobacco leaves as described in the Experimental section, and tested for oxidative activity at various concentrations of substrate and malonate. When citrate, α -oxoglutarate or malate was substrate the cofactors listed in Table 8 were present. Inhibitions of oxidative activity are calculated from the reductions in q_{O_2} values.

Substrate	Substrate concn. (mM)	Ratio: substrate/malonate	Inhibition (%)
Succinate	112.0	30	31
	25.0	6.6	68
	3.8	1.0	100
Citrate	27.0	1.3	45
	2.7	0.13	62
α -Oxoglutarate	2.5	0.15	68
	1.3	0.074	70
Malate	27.0	1.3	2
	2.7	0.13	0

Effect of inhibitors on organic acid oxidations

Malonate inhibited tobacco-leaf succinoxidase (Table 9), and the variation of the inhibition with the ratio of substrate to inhibitor suggests a competitive inhibition. The oxidation of at least two other substances of the tricarboxylic acid cycle, citrate and α -oxoglutarate, was inhibited by malonate although to a smaller extent than succinate. Malate oxidation was not at all affected. Presumably the amount of inhibition is a measure of the proportion of the oxygen uptake in these reactions which was mediated by succinic dehydrogenase.

Potassium cyanide was also a potent inhibitor of succinoxidase. At a concentration of 10 μ M it inhibited by 75 %, and at 0.1 mM inhibition was virtually complete. In these experiments the KOH in the Warburg flasks was replaced by the KOH-KCN mixtures designed to keep the concentration of cyanide in the incubation medium constant (Umbreit *et al.* 1949). Although this method is less satisfactory than those with $\text{Ca}(\text{CN})_2$ - $\text{Ca}(\text{OH})_2$ mixtures, Simon (1957) found that the error involved in using it was small.

The succinoxidase was less strongly inhibited by sodium fluoride, a concentration of 12.5 mM being necessary for a 40–50 % reduction in activity. It was not appreciably affected by diethyldithiocarbamate at concentrations up to 2 mM.

Citrate content of tobacco leaves

The suspensions made by grinding tobacco leaves in the extraction medium from which citrate had been omitted had a citrate concentration corresponding to 15–20 m.-equiv. of citrate/kg. fresh weight of leaf. This agrees well with values found by Vickery & Palmer (1957). On fractionation of the suspensions the great bulk (90–95 %) of this citrate went into the supernatant fraction. Very little (0.5–1.5 %) was associated with either the chloroplast or mitochondrial fractions. This distribution was not altered by the inclusion of succinate, which has a stabilizing effect on the mitochondrial fraction succinoxidase (Table 3), in the extraction medium.

The amount of citrate released on disruption of the leaves was presumably too little to have maximal stabilizing effect on the succinoxidase. The citrate added in the extraction medium was usually five or six times this amount.

DISCUSSION

Cytoplasmic particles, whose appearance and staining properties suggest that they are mitochondria, have been identified in leaves and other tissues of tobacco plants with both light (McGivern,

1957) and the electron microscope (Pallade, 1953). They have also been identified in extracts made by grinding tobacco leaves in a sucrose-phosphate medium (McClendon, 1952, 1953; Jagendorf & Wildman, 1954), where they were associated with cytochrome oxidase. None of these extracts, however, would bring about the oxidation of organic acids of the tricarboxylic acid cycle. Jagendorf & Wildman (1954) attributed this to unfavourable conditions in the isolation process.

The results of the present work bear out this conclusion. Grinding the leaves exposes the enzyme systems to the action of acids and other inactivators contained in other compartments of the cells. Even when the leaves are ground in large amounts of extraction media, transient high concentrations of deleterious substances are likely to occur. Active systems which bring about the oxidation of organic acids by the reactions of the tricarboxylic acid cycle were only prepared in the presence of citrate and large amounts of buffer. The full nature of the protective action of these substances is not understood, but it is evident that the protection was not complete. The requirement for added cytochrome *c* suggests some degree of mitochondrial damage (Miller, 1953), and the low citrate content may do so also (Schneider *et al.* 1956). Moreover a satisfactory 'sparked' oxidation of pyruvate was obtained only when the leaf acids were neutralized by ammonia infiltration before grinding.

In a recent review (Steinberg & Tso, 1958) it was pointed out that not all the enzymes responsible for catalysing the tricarboxylic acid cycle have been detected in tobacco plants. The present evidence greatly supplements the data, obtained by Glock (1956) on etiolated tobacco seedlings, and suggests that the cycle could operate in intact leaves. Consistent with its operation are the metabolic relationships between the organic acids of cultured, excised leaves (Vickery & Palmer, 1957) and the light-reversed carbon monoxide inhibition of tobacco-leaf respiration (Kempner, 1936). But the proportion of the total respiration of the leaves which is mediated by the cycle is unknown.

SUMMARY

1. A succinoxidase preparation was made from tobacco leaves by grinding them in a medium containing sucrose, 2-amino-2-hydroxymethylpropane-1:3-diol, citrate, phosphate and ethylenediaminetetra-acetic acid, and then centrifuging between 1000 and 10 000 g. Omission of any one of the components of the medium, except phosphate, brought about a decrease in succinoxidase activity which was greatest for citrate and decreasingly less for 2-amino-2-hydroxymethylpropane-1:3-

diol, sucrose and ethylenediaminetetra-acetic acid.

2. These preparations contained the bulk of the succinoxidase of the leaf extract together with a third of the total chlorophyll and 15 % of the total protein nitrogen. Only a third of this sedimented protein nitrogen, corresponding to about 5 % of the protein nitrogen of the extract, was of non-chloroplast origin.

3. The conditions necessary for measuring maximal activity of these succinoxidase preparations included the addition of cytochrome *c*, sucrose and Mg^{2+} ions.

4. The oxidation of succinate was inhibited reversibly by malonate. It was inhibited strongly by cyanide, less strongly by fluoride and not at all by diethyldithiocarbamate.

5. The preparations oxidized most of the acids of the tricarboxylic acid cycle; some of these oxidations were stimulated by mixtures of diphosphopyridine nucleotide, thiamine pyrophosphate and coenzyme A. A 'sparked' oxidation of pyruvic acid was obtained only if the leaves were vacuum-infiltrated with dilute ammonia before being disintegrated.

6. The products formed during the oxidation of these acids were other acids of the tricarboxylic acid cycle.

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The Action of Certain Drugs on the Uptake of ^{32}P by Human Erythrocytes

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Apart from the uptake of a small fraction which is rapidly labelled, the uptake of phosphate by erythrocytes is a temperature-dependent, probably enzymically regulated, process (Gourley & Gemmill, 1950). There is some doubt whether the phosphate is first incorporated into the labile radicals of adenosine diphosphate and adenosine triphosphate (Gerlach, Fleckenstein & Gross, 1958) or is transferred to these molecules from diphosphoglycerate (Pranker & Altman, 1954). The energy for this uptake is generally attributed to glycolysis. A search has been made for suitable agents which might block the uptake of ^{32}P by interfering with the formation of either adenosine triphosphate or 2:3-diphosphoglycerate. The results of these investigations are reported in this paper.

METHODS

Action of drugs on phosphate exchange. Blood was obtained by venepuncture from healthy adult males. Clotting was prevented with solid heparin (Pularin brand, Evans Medical Supplies Ltd., 0.5 mg./ml. of blood). For each observation, 1 ml. of blood was added to 10 ml. of the following medium: NaCl 142 mM, KCl 5.9 mM, MgSO_4 1.5 mM, CaCl_2 1.7 mM, NaH_2PO_4 0.5 mM, Na_2HPO_4 2.8 mM,

dextrose 5.6 mM. Drugs were incorporated into the medium as required and when necessary the pH was restored to 7.4 with NaOH or HCl. pH was measured with a Universal pH meter (W. G. Pye Ltd.) calibrated with standard buffer solutions (National British Standard formula) pH 6.99 and 9.15. All treatments were carried out in duplicate and compared with a pair of untreated controls in each experiment. Immediately after adding the blood to the medium the flasks were incubated in a water bath at 37°. After 60 min., 0.2 ml. samples of carrier-free ^{32}P -phosphate were added to each flask and after a further 60 min. the contents of the flasks were poured into centrifuge bottles at 0° and were centrifuged for 10 min. at 1200 g in a refrigerated centrifuge. The supernatants were removed, the cells were haemolysed and digested with 2.2 ml. of 60% (v/v) perchloric acid for 2 hr. The total phosphate and ^{32}P in the digest were then estimated. To find the specific activity of the whole suspension, 0.2 ml. samples of ^{32}P -phosphate were added to two further controls. These were left at -20° in order to haemolyse the cells. Samples were then taken and treated as above.

Estimation of total phosphorus. Phosphorus was estimated by Allen's (1940) method. The intensity of the blue colour, produced by the reduction of phosphomolybdic acid by 2:4-diaminophenol hydrochloride, was measured with a type H 760 Spekker absorptiometer (Hilger and Watts Ltd.) with Ilford 608 filters (maximum transmission 83% at 7000 Å). Cells 0.25 or 4.0 cm. long were used, depending on the concentration of phosphorus being estimated.

Estimations of radioactivity. ^{32}P was estimated with a liquid Geiger-Müller counter (type M6, 20th Century Electronics Ltd.) and conventional counting equipment (Dynatron Radio Ltd.). The counts were corrected for background and for lost counts due to the resolving time of

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