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MITOCHONDRIAL PREPARATIONS FROM THE FRUIT OF THE APPLE—I.

PREPARATION AND GENERAL ACTIVITY

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Abstract—A fraction has been prepared from apple fruits and shown to have many of the properties generally ascribed to "mitochondria". It is considered that the activity of such preparations, which can be obtained with a degree of reproducibility, quantitatively represents the mitochondrial activity in tissue from fruit in a "steady" physiological state and warrants their use in studies of changes in mitochondrial activity with changing physiological states.

INTRODUCTION

BEGINNING with the work of Pearson and Robertson,[1] particulate fractions containing many of the enzymes of the Krebs cycle have been isolated from the tissue of mature apple fruits. Although the activity of the earlier preparations was lower than that of mitochondrial preparations from other plant tissues, they had many of the properties of authentic mitochondria. More recently, Tager,[2] Lieberman[3–5] and Hatch *et al.*[6] have obtained from the pulp of mature apples more active preparations which contain cytochromes a, a_3, b and c, and have the capacity for oxidative phosphorylation. Jones and Hulme[7] have given a preliminary report of a new method for preparing a mitochondrial fraction of considerably greater activity. The new method, which it is the purpose of the present paper to describe in detail, gives particles having at least three times the activity of earlier preparations from the apple fruit and comparable with the highest activities obtained from leaves.

In the apple fruit the high acidity of the tissue has long been known as an obstacle to the isolation of enzymes from it, but it has recently become clear that, in addition, phenolic compounds (leucoanthocyanidins, catechins, quercetin and cyanidin glycosides) present in the fruit (Williams[8,9] and Siegelman[10]), especially in the peel, are largely responsible for the low activities previously reported for mitochondria isolated from apples. The failure of earlier workers to obtain active particles from very young fruits is probably due to their very high "tannin" content. The reduction in activity by phenolic compounds appears to be brought about in two ways. Firstly by direct inhibition, and secondly by "co-precipitation"

[1] J. A. PEARSON and R. N. ROBERTSON, *Australian J. Biol. Sci.* 7, 1 (1954).
[2] M. TAGER, *Nature, Lond.* 182, 1521 (1958).
[3] M. LIEBERMAN, *Science* 127, 189 (1958).
[4] M. LIEBERMAN, *Plant Physiol.* 35, 796 (1960).
[5] M. LIEBERMAN, *Plant Physiol.* 36, 804 (1961).
[6] M. D. HATCH, J. A. PEARSON, A. MILLERD and R. N. ROBERTSON, *Australian J. Biol. Sci.* 12, 167 (1959).
[7] J. D. JONES and A. C. HULME, *Nature, Lond.* 191, 370 (1961).
[8] A. H. WILLIAMS, *Ann. Rept. Agr. Hort. Research Sta. Long Ashton, Bristol*, p. 219 (1952).
[9] A. H. WILLIAMS, *Phenolics in Plant in Health and Disease*, p. 3, Pergamon Press, London (1960).
[10] H. W. SIEGELMAN, *J. Biol. Chem.* 213, 647 (1955).

with the mitochondria of inactive (in the mitochondrial sense) protein complexed with phenolic material in various stages of oxidation and polymerization. Chlorogenic acid, which is abundant in apple fruit,[11] has been reported to inactivate completely mitochondria from sweet potatoes (Lieberman and Biale[12]). We find, however, that apple mitochondria oxidize succinate almost equally well in the presence or in the absence of this phenolic compound (Hulme and Jones[13]). We have some evidence that leaf tissue containing various phenolic compounds also yields mitochondrial preparations of low activity unless steps are taken to remove these "tannins" during the isolation procedure (Hulme and Jones[13]).

Our new method employs polyvinylpyrrolidone (PVP) in the extraction medium to mitigate the inhibitory action of phenolic compounds. Nylon powder ("Perlon") was found to be ineffective in this respect.

RESULTS

Details of the preparation of the mitochondrial suspensions and of the methods used to determine the activity of the various enzymes present in them are given in the Experimental Section. These suspensions will be referred to throughout as "mitochondria" although it is certain that they contain some unidentified debris. An evaluation of the integrity of the mitochondria will be made later. Except where stated otherwise, the concentration of PVP used throughout the present paper was usually 0·75% (w/v) since it had been found that this gave optimal results for the stage of maturity of the Cox's Orange Pippin apples used, i.e. picked in the immediate pre-climacteric state and used immediately or after a period of storage at 2·8°. A subsequent paper in this series will be concerned with the effect of the concentration of PVP used in the extraction medium on the polyphenols present and on the activity of the mitochondria. It is essential that pharmaceutical grade PVP is used; commercial grades contain material which inactivates enzyme systems. Throughout the present work, the final pH of the medium and tissue macerate was 7·3–7·5.[7]

Figure 1 shows an electron-micrograph made from a mitochondrial pellet prepared from apple peel using PVP in the extracting medium. The double membrane and tubules (cristae) are clearly visible and the mitochondrion is very similar in appearance to plant mitochondria generally.[14]

Oxidative Decarboxylation of Succinate and Malate

It should be pointed out here that, unless a "stage inhibitor" is used, the O_2-uptake and the CO_2-output in response to a given substrate of the Krebs cycle is only primarily the result of action on the added substrate; subsequent steps in the cycle are involved in the overall O_2-uptake and CO_2-output.

(*a*) *Effect of the presence of PVP in the extraction medium.* Figure 2 gives the O_2-uptake with succinate as substrate of mitochondria prepared from whole fruits with and without PVP (5% w/v) in the extraction medium. It will be seen that the endogenous O_2-uptake is appreciable when PVP is not used. Table 1 gives the activity of mitochondria prepared from peel and pulp tissue without and with the use of PVP. If these results were expressed in terms of the nitrogen (protein) content of the preparations, the difference between with and without

[11] A. C. HULME, *Biochem. J.* **53**, 337 (1953).
[12] M. LIEBERMAN and J. B. BIALE, *Plant Physiol.* **31**, 420 (1956).
[13] A. C. HULME and J. D. JONES, *Enzyme Chemistry of Phenolic Compounds*, p. 97, Pergamon Press, London (1963).
[14] A. B. NOVIKOFF, *The Cell, Vol. II*, p. 299, Academic Press, New York (1961).

PVP would be enhanced, since, as had been stated elsewhere (Hulme and Jones[13]), if polyphenols are not removed by PVP they bring down with the mitochondria large amounts of non-mitochondrial protein. For example the nitrogen contents (nitrogen insoluble in trichloracetic acid) of the third and fourth samples were 1·48 mg and 0·28 mg (per 10 g original tissue) respectively, and of fifth and sixth, 1·29 and 1·03 mg respectively. The effect of PVP

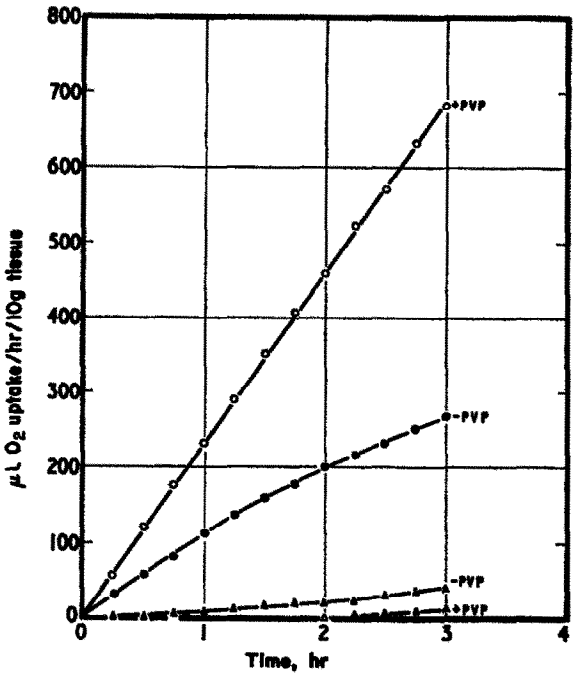

FIG. 2. THE O_2-UPTAKE IN THE WARBURG RESPIROMETER OF A MITOCHONDRIAL DIGEST WITH (○, ●) AND WITHOUT (△, ▲) THE PRESENCE OF SUCCINATE AS SUBSTRATE AND WITH OR WITHOUT THE PRESENCE OF PVP IN THE EXTRACTION MEDIUM. See text.

TABLE 1. MITOCHONDRIAL ACTIVITY OF PREPARATIONS FROM PEEL AND PULP WITH OR WITHOUT THE USE OF PVP IN THE EXTRACTING MEDIUM. AVERAGE OF 2-HR RUN IN WARBURG RESPIROMETER

		Activity/hr/10 g tissue (μl gas)			
		Substrate–succinate		Substrate–malate	
Tissue	PVP (%)	O_2-uptake	CO_2-output	O_2-uptake	CO_2-output
Peel (pre-climatic rise)	0	35	0	—	—
	0·75	225	61	—	—
Peel (during climacteric rise)	0	160	3	92	68
	5	650	194	164	84
Peel (from cold storage)	0	146	13	24	15
	0·75	743	248	341	231
Pulp (from cold store)	0	106	28	32	45
	0·75	492	122	184	162

in greatly reducing the co-precipitation of tannins with the mitochondrial preparations will be seen from Table 2.

(*b*) ***Persistence and reproducibility of the reactions.*** The course of the oxidation and

Table 2. Tannin content of various mitochondrial preparations

Tissue	Concentration of PVP in extraction medium (%)	Tannin (mg/0·5 ml suspension)		
		Total	Leucoanthocyanins	Flavan
Peel	0	1·23	1·01	0·268
	0·75	0·454	0·605	0·135
	5·0	0·113	0·080	0·031
Pulp	0	0·616	0·805	0·159
	0·75	0·110	0·125	0·028

decarboxylation of succinate over a 4-hr period by mitochondria prepared from peel and pulp is shown in Fig. 3. The O_2-uptake and CO_2-output are linear for the whole 4-hr period. This is usual with apple mitochondria, although a fall-off is found when very senescent tissue is

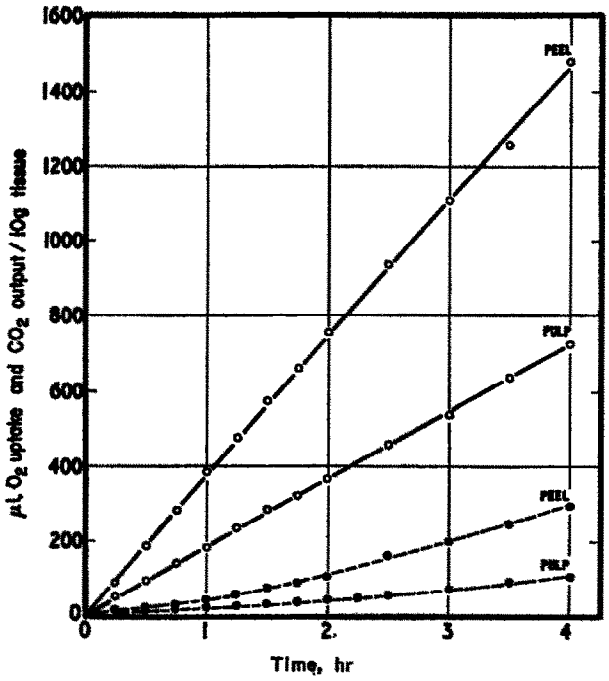

Fig. 3. The O_2-uptake (○) and CO_2-output (●) of mitochondrial preparations from the peel and pulp of apple fruits with succinate as substrate.

used. The reproducibility of the preparative procedure in relation to the oxidative decarboxylation of the malate and succinate is shown in Table 3 for periods when the respiratory activity of the whole fruit is remaining at a fairly constant level. The preparations in each

category (pre- and post-climacteric) were made, over a period of fourteen days, from fruit developing on the tree. Results for replicate preparations from stored fruits on successive days agree even more closely (Hulme, Jones and Wooltorton[15]).

TABLE 3. MITOCHONDRIA ACTIVITY OF REPLICATE PREPARATIONS FROM APPLE PEEL AND PULP USING 5% PVP IN THE EXTRACTION MEDIUM. (AVERAGE OF 2 HR RUN IN WARBURG RESPIRATOR; CORRECTED FOR SMALL ENDOGENOUS RESPIRATIONS)

	Activity/hr/10 g tissue (μl gas)			
	Substrate–succinate		Substrate–malate	
Tissue	O_2-uptake	CO_2-output	O_2-uptake	CO_2-output
Peel (pre-climacteric fruit)	190	38	100	78
	193	32	93	70
	192	35	90	67
Pulp (pre-climacteric fruit)	220	52	97	72
	198	40	78	68
	217	48	93	71
Peel (post-climacteric fruit)	372	52	137	80
	333	55	123	78
	360	63	139	79
Pulp (post-climacteric fruit)	181	22	89	65
	172	37	78	61
	159	38	69	54

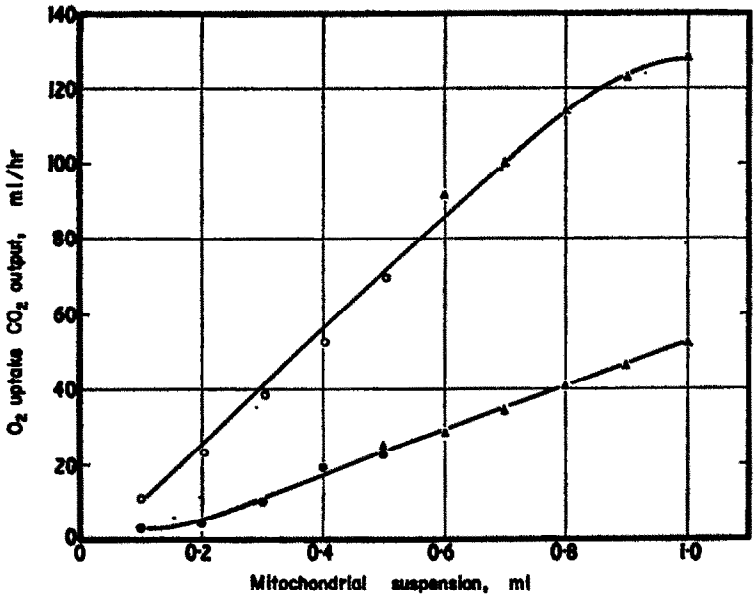

FIG. 4. THE O_2-UPTAKES AND CO_2-OUTPUTS OF MITOCHONDRIAL DIGESTS WITH SUCCINATE AS SUBSTRATE USING VARYING AMOUNTS OF MITOCHONDRIAL SUSPENSION. The circles represent one preparation and the triangles a second preparation. Open symbols represent O_2-uptake and filled-in symbols represent CO_2-output.

[15] A. C. HULME, J. D. JONES and L. S. C. WOOLTORTON, *Proc. Roy. Soc. B.* **158**, 514 (1963).

TABLE 4. MITOCHONDRIAL ACTIVITY OF PREPARATIONS FROM PEEL, PULP AND WHOLE FRUIT TISSUE WITH VARIOUS SUBSTRATE ACIDS OF THE KREBS' CYCLE. AVERAGE OF THE FIRST 2 HR

Tissue	Activity/hr/10 g tissue (μl gas, O_2-uptake or CO_2-output*)																			
	Citrate		Cis-aconitrate		Isocitrate		Oxalo-succinate†		α-oxo-glutarate		Succinate		Fumarate		Malate		Pyruvate		Pyruvate+ Malate	
Peel (Cox's O.P.)	134	(130)*	230	(260)	205	(176)	100	(92)	254	(194)	736	(178)	182	(148)	340	(281)	16	(268)	156‡	(340)
Peel (King Edward)											430	(80)			218	(154)	12	(115)	84‡	(156)
Pulp (Cox's O.P.)	49	(48)	74	(86)	60	(52)	70	(51)	72	(58)	245	(60)	52	(47)	92	(68)	10	(46)	47‡	(74)
Whole fruit (Cox's O.P.)	44	(38)							75	(61)	180	(16)	36	(27)	70	(49)				

* CO_2-output (in brackets).
† Corrected for non-enzymic breakdown.
‡ "Sparking" amount of malate (2 μmole) with pyruvate (40 μmole). Allowance made for small malate oxidation.

(*c*) *Effect of the amount of mitochondrial suspension.* Results for various amounts of mitochondrial suspension within the standard Warburg assay conditions are shown in Fig. 4. The O_2-uptake over the first two hours shows a linear increase between 0·1 and 0·8 ml of mitochondrial suspension and the subsequent falling-off in rate with larger amounts is probably due to depletion of substrates. The average value of the CO_2-output for the first two hours is linear with an amount of suspension between 0·2 and 0·8 ml. The low CO_2-output with the smallest amounts of mitochondria may be due to lack of sufficient enzyme to carry the reaction appreciably beyond the oxidation of succinate to fumarate.

Oxidative Decarboxylation of Other Acids of the Krebs Cycle

Examples of the action of the mitochondria on various acid substrates are given in Table 4. Oxaloacetic has been omitted because of the difficulty in preventing its spontaneous decarboxylation. The variations in the activity with succinate are explained by the fact that the mitochondria were prepared from fruit at different physiological stages of maturity. It is interesting to note that where the substrate is at or very near a decarboxylation stage (e.g. oxalosuccinic, α-oxoglutarate and isocitrate) the ratio of the CO_2 evolved to the O_2 taken up (the R.Q.) is close to unity.

TABLE 5. RELATIVE CONCENTRATIONS OF ORGANIC ACIDS FORMED DURING A 2-HR RUN IN THE WARBURG RESPIROMETER OF MITOCHONDRIA FROM APPLE PEEL

Original substrate	Acids found in digest				
	Citrate	Oxalosuccinate	Succinate	Fumarate	Malate
Succinate	+	−	++++	++	+++
Malate	++	−	−	++	++++
Citrate	++++	+	−	−	−

Pyruvate on its own appears to be broken down entirely by carboxylase, since there is virtually no O_2-uptake. The situation is different when "sparking" amounts of malate are also present. The degree to which utilization of pyruvate becomes divided between carboxylase and the Krebs cycle here cannot be decided precisely on the present data; the high rate of O_2-uptake, however, suggests that a large part is metabolized via the cycle.

In common with the total respiration of whole tissue (Hulme[16]), the activity of the mitochondria from peel is 3 to 4 times greater than that of mitochondria from pulp.

Paper chromatograms of the organic acids prepared after a 2-hr run in the Warburg gave the results shown in Table 5. No acids were present on the chromatograms other than those listed in the table.

Chromatograms of the 2:4 dinitrophenylhydrazones of the keto acids present in the digest after a 2-hr run in the Warburg respirometer with succinate (4·72 mg) as substrate showed that the amounts of the keto acids present were: Pyruvic acid (including isomer), 0·138 mg; and α-oxoglutaric acid, 0·078 mg, and a trace of oxaloacetic acid.

[16] A. C. HULME, *Advances in Horticultural Science and Their Applications*, p. 77, Pergamon Press, London (1961).

The Dehydrogenases

The activities of malic dehydrogenase, diaphorase and cytochrome-c reductase as a function of the quantity of mitochondrial suspension are shown in Fig. 5. Table 6 gives the relative activities of various dehydrogenases; these results are typical for our apple mitochondria. It is interesting that, although succinic dehydrogenase activity was so low, the coupled succinic-cytochrome-c reductase activity of the same preparations was quite high (Table 6). This low activity in direct measurement of succinic dehydrogenase by the method used here is not unusual with plant mitochondria (Pierpoint, personal communication). Use

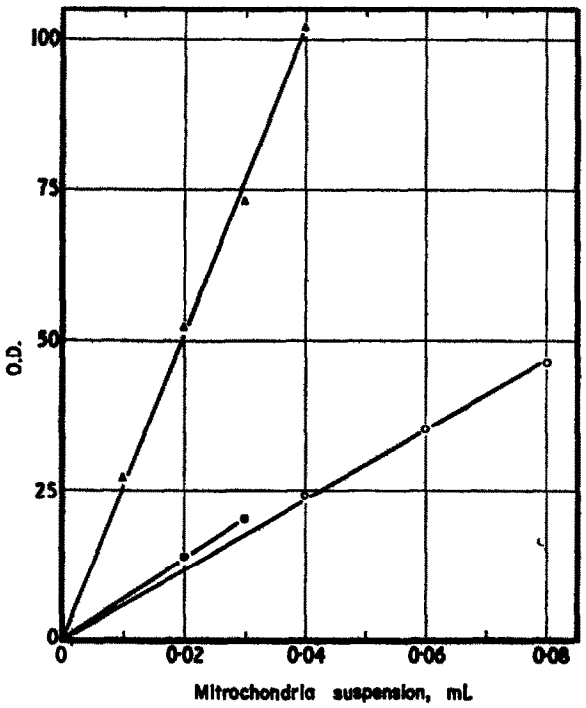

FIG. 5. ACTIVITY OF MALIC DEHYDROGENASE (○), CYTOCHROME-C REDUCTASE (●) AND DIAPHORASE (△) WITH INCREASING AMOUNTS OF MITOCHONDRIAL SUSPENSION.

of PMS as an additional electron carrier increased the "activity" of both malic succinic dehydrogenase, but it had no relative effect on activity.

The results show that the activities of one dehydrogenase in relation to another and to the "overall" O_2-uptake of the complete system in the Warburg respirometer varies with the physiological state of the tissue.

The Effect of Sonication on the Mitochondria

After sonication, even at the shortest period of 5 sec, a profound change in the physical state of the mitochondria was obvious. After centrifugation of the sonicated suspension, a yellow oily layer, which very soon became dispersed, appeared on the surface of the green supernatant liquid. The pellet, instead of being bright green like the original mitochondria, was almost colourless.

TABLE 6. THE ACTIVITY OF VARIOUS DEHYDROGENASE IN MITOCHONDRIA PREPARED FROM PEEL OF APPLES IN VARIOUS PHYSIOLOGICAL STATES

Fruit	P.V.P. in extracting medium (%)	Dehydrogenase activity (units/10 g original tissue—see text)					O_2-uptake in Warburg respirator (μl/10 g original tissue)	
		Succinic	Malic	Diaphorase	Cytochrome-c reductase	Succinic–cytochrome-c reductase	Succinate	Malate
Fresh; pre-climacteric	0	0	10	140	0	—	48	—
	0·75	10	160	1730	2650	—	360	—
Ditto	0·75	14	98	1480	3250	—	224	—
Ripe, cold stored	0	19	60	160	10	23	148	—
	0·75	50	570	1340	1800	420	612	—
	5·0	48	330	820	900	300	446	—
Ditto	0	51	54	157	416	—	152	24
	0·75	61	325	2050	3250	—	760	368

The results obtained for the determination of succinic and malic dehydrogenases and diaphorase in the supernatant liquid are shown in Fig. 6*b*. In Fig. 6*c* are shown the activities of the three enzymes in the re-suspended sonicated particles. Fig. 6*a* shows the effect of sonication on the O_2-uptake in the Warburg respirometer of the mitochondria.

While sonication rapidly decreases the activity of the complete succinoxidase system, and to a less extent the oxidative decarboxylation of malate, there is a small increase in malic

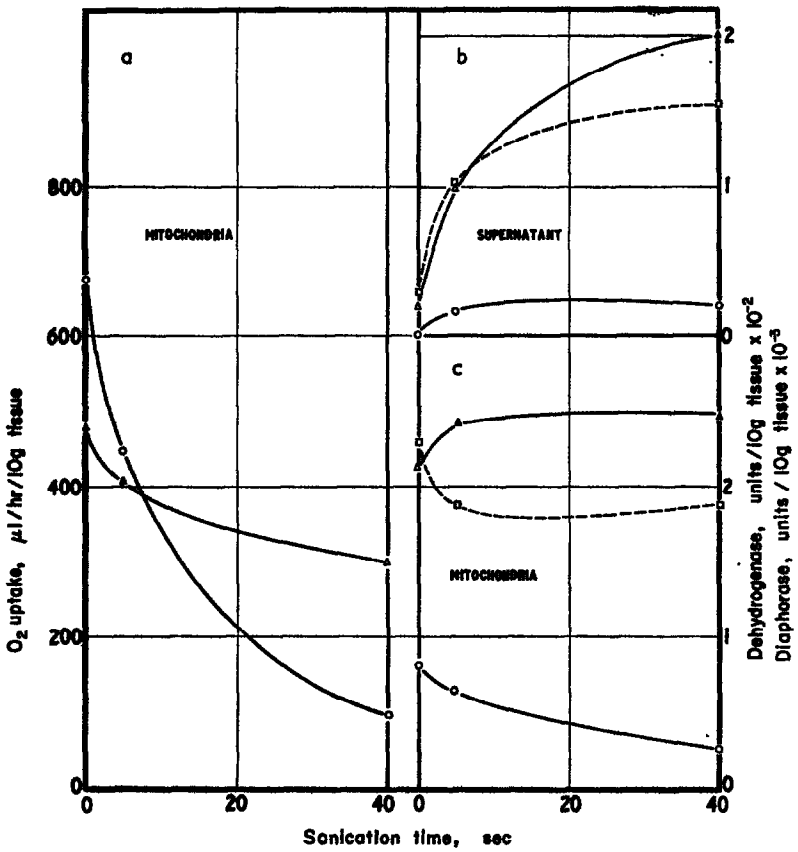

FIG. 6. ACTIVITY OF ENZYMES IN SONICATED MITOCHONDRIA AND THE SUPERNATANT LIQUID FROM SONICATED MITOCHONDRIA. See text.

(*a*) O_2-uptake in Warburg respirometer with succinate as substrate (○) and with malate (△) as substrate; (*b*) and (*c*) dehydrogenases: ○, succinic dehydrogenase; △, malic dehydrogenase; □, diaphorase.

dehydrogenase, although succinic dehydrogenase and diaphorase decrease. The most striking effect is the rapid transfer of malic dehydrogenase and diaphorase from the particulate fraction to the supernatant fraction; succinic dehydrogenase also appears in this latter fraction.

The total nitrogen content of the supernatant fraction increases from 0·081 mg/0·5 ml for the supernatant fraction of the unsonicated mitochondrial suspension to 0·195 mg after 5 sec sonication and 0·208 mg after 40 sec sonication. The total nitrogen of the original mitochondrial suspension was 0·341 mg/0·5 ml (0·238 mg TCA insoluble N/0·5 ml).

DISCUSSION

The main purpose of the present paper is to describe in detail a method for the preparation of mitochondria from plants, especially apple fruits, which have acid saps and contain relatively large amounts of readily oxidizable polyphenols such as catechins and leucoanthocyanins. Experience leads us to believe that while some of these phenolic compounds may, especially if carried through to the final mitochondrial preparations, cause direct inhibition of the mitochondrial enzymes, a more serious effect is the general combination of these compounds, particularly when oxidized and polymerized, with protein and other high molecular weight compounds to give inactive agglomerates. The object has not been to prepare a minimum of highly "purified" intact mitochondria, but to obtain a maximum (constant) amount of "mitochondrial fraction", capable of being used as a reasonable measure of mitochondrial activity during the general ontogeny of the fruit and during important changes in physiological state such as the respiration climacteric. These changes, as well as specific attributes of these mitochondrial preparations, will be described in subsequent papers.

The first obvious indication that the incorporation of PVP in the extraction medium has prevented oxidation of the polyphenols, probably by combination with the unpolymerized

TABLE 7. ACTIVITY IN TERMS OF O_2-UPTAKE WITH SUCCINATE AS SUBSTRATE OF MITOCHONDRIA PREPARED BY VARIOUS METHODS OF TISSUE DISINTEGRATION (5% PVP PRESENT IN EXTRACTION MEDIUM THROUGHOUT)

Method of maceration	Activity (average of three preparations)			
	μl O_2/hr/mg total N	μl O_2/hr/mg TCA insol N	q O_2 (μl O_2/hr/mg protein)	μl O_2/10g/tissue
Roller Mill	790	1056	169	647
Virtis blendor ($\frac{1}{2}$ speed)	182	240	39	175
Atomix blendor ($\frac{1}{2}$ speed)	204	295	47	91
Grinding with sand	342	507	81	287
Grinding with alumina	474	545	87	352

compounds to give a soluble complex, lies in the bright green colour of the extracts and subsequent preparations. We have found that the incorporation of ascorbic acid, cysteine, etc. (without PVP), gives only a transient protection against the oxidative darkening which occurs very rapidly if no "protective" action is taken. Without PVP the final mitochondrial preparations are dark brown, flocculent and bulky.

The advantages of the roller mill method over the more conventional use of Waring blendor types of macerator, or grinding with sand and alumina have been discussed elsewhere;[17] some results are given in Table 7. The reasons for expressing activities in terms of original tissue rather than nitrogen or protein have also been discussed (Hulme, Jones and Wooltorton[15]). It should be emphasized, however, that our method, in addition to giving higher yields of "mitochondria", does not necessarily give a poorer product in terms of nitrogen or protein content (whatever that may mean in terms of "true" mitochondrial nitrogen) i.e. in terms of absolute activity (see Table 7).

It is realized that all these preparations represent a crude mitochondrial fraction and are far from being preparations consisting entirely of unbroken, undamaged organelles. Nevertheless, the direct evidence provided by the electron-micrograph shown in Fig. 1 establishes

[17] J. D. JONES and A. C. HULME, *Biochem. J.* **88**, 44 (1963).

that, in our pellets, there are at least some intact mitochondria, complete with double membranes and having a very similar appearance to those seen in intact tissue. We consider that the data presented, including the effects of sonication of our mitochondrial preparations, provide strong evidence that intact mitochondria can now be readily obtained from apple fruit tissue having all the properties of, and not differing in any essential way from, mitochondria which have been obtained from other plant material by a growing number of workers. Finally we should point out that the persistence of the oxidative decarboxylation of succinate and malate at a high level in the Warburg studies over a period of 4 hr is unusual with plant mitochondria.

EXPERIMENTAL

Apple Fruits Used

Cox's Orange Pippin apples from 50 trees grown on Malling IX rootstocks in a grassed-down orchard were used for most of the experiments; a few experiments employed Edward VII apples from cold storage.

Mitochondrial Preparations

All manipulations, except centrifugation, were performed in a room at 1°: centrifugations were carried out in refrigerated centrifuges.

Twenty-five grammes of peel, removed from the fruit with a stainless steel domestic potato peeler, was added immediately piece by piece to 120 ml of extraction medium (see below) contained in a crystallizing dish. A close-fitting perforated polythene disk was placed over the peel in the dish to keep it below the surface of the liquid, and the dish was put into a small vacuum desiccator. Vacuum was applied and maintained for 10 min during which time the desiccator was gently shaken to allow all the air in the tissue to escape and be replaced by extraction medium. The vacuum was released, the polythene disk removed from the dish, and the liquid poured into a larger (15 cm dia.) dish which was placed on the magnet-platform of a magnetic stirrer and under the roller of a special stainless steel mill (Jones[18]). The rotor of the magnetic stirrer was placed in the liquid and run at a slow speed while the peel strips were fed through the rollers of the mill with plastic forceps. As the peel was passed through the rollers it was continually bathed with further extraction medium (80 ml) from a plastic wash-bottle. Thus 200 ml in all of extraction medium was used per 25 g peel. The composition of this medium was: Sucrose 0·4 M; 2-amino-2-hydroxymethylpropane-1:3-diol (tris) 0·2 M; citrate 0·02 M (Pierpoint[19]); KH_2PO_4 0·01 M; ethylenediaminetetraacetic acid (EDTA) 0·01 M. The pH of this medium was made to 7·7–7·8 with H_3PO_4, depending on the maturity of the fruit. When polyvinylpyrrolidone (PVP) was used, the requisite amount was dissolved in the medium. The pH of the macerate was 7·3–7·5.

The macerated peel in the extraction medium was then filtered and squeezed through fine cloth and the filtrate centrifuged at 1000 *g* for 10 min. The supernatant was further centrifuged at 15,000 *g* for 20 min and the green "mitochondrial pellet" obtained was washed by resuspension in 20 ml of a solution containing sucrose (0·4 M) and EDTA (0·01 M) made to pH 7·5 with KOH, using a Duall glass homogenizer with a "Teflon" pestle driven by a slow-speed (400 rev/min) motor. After separation by centrifugation at 15,000 *g* for 10 min the pellet was suspended in 5·0 ml 0·2 M sucrose using the homogenizer. This suspension is the "mitochondrial preparation". In some early experiments, the suspending medium also contained potassium phosphate (0·05 M, pH 7·5) and small amounts of ATP and AMP but

[18] J. D. JONES (in press).
[19] W. S. PIERPOINT, *Biochem. J.* 71, 518 (1959).

this did not materially affect the subsequent activity of the particles and the practice was discontinued.

Oxygen Uptake and Carbon Dioxide Output

These were measured, at 25°, in the conventional Warburg respirometer by the methods described by Umbreit, Burris and Stauffer.[20] The shaking rate was 110 strokes/min and equilibration was carried on for 5 min before the taps were closed. The flasks contained Sucrose 400 μmole; KH_2PO_4 adjusted to pH 7·5 with KOH, 37·5 μmole; $MgSO_4$ 10 μmole; $MnSO_4$ 0·1 μmole; cytochrome-c 0·018 μmole; crystallized bovine plasma albumin 3 mg; yeast concentrate 2 mg; substrate acid adjusted to pH 7·5 with KOH, 40 μmole; mitochondrial preparation 0·25–0·5 ml, and water to make to 2 ml. Controls without substrate were always performed. In some early experiments with succinic acid as substrate, yeast concentrate was replaced by an appropriate mixture of ATP, NAD, thiamine pyrophosphate chloride and CoA. Generally similar results were obtained although occasionally the yeast concentrate gave slightly higher activities.

Results were usually expressed as μl gas/hr/10 g original tissue averaged over the first two hours. The expression of activity in terms of protein N was only used in special cases because of the difficulty experienced in obtaining a value for the true nitrogen content of the preparation. This has been discussed in detail elsewhere (Hulme, Jones and Wooltorton[15]).

Succinic and Malic Dehydrogenases

The activity of these enzymes was measured by following the decrease in absorptivity at 600 mμ due to the coupled reduction of the dye 2,6-dichlorophenolindophenol (DCPIP). Absorptivity changes were measured in 1-cm cuvettes containing the following: 1 ml composite solution (A) containing buffer and dye (see below); 0·2 ml NAD (1·1μmole); 0·01–0·1 ml mitochondrial preparations; 0·1 ml (20 μmole) acid substrate and water to make to 3·0 ml. Solution A contained, per ml: 60 μmole potassium phosphate (pH 7·3); 6 μmole KCN; 0·186 μmole DCPIP; 10 μmole nicotinamide; 16 μmole magnesium sulphate. It was prepared fresh each day from stock solutions of the various compounds which were stored at 1°, except for the KCN solution which was freshly prepared each day. Measurements of absorptivity were taken immediately on the addition of substrate and at 30-sec intervals for 2–3 min. The reaction mixture without substrate was used as blank. The rate of the reaction was linear for at least 2 min. One enzyme unit was taken as the amount of mitochondrial preparation which caused a corrected initial rate of change in absorptivity of 0·01/min under the above conditions, "initial rate" being the rate measured between 30 and 90 sec from the addition of substrate. Specific activity equals enzyme units/10 g original tissue; a nitrogen (protein) basis was not used for specific activity for the reasons given above. All spectrophotometric measurements were made at 20°, and an amount of mitochondrial preparation was always used so that changes in absorptivity per 30 sec lay between 0·03 and 0·06. In some experiments the use of phenazine methosulphate (PMS) as an intermediate electron carrier was tried. Although this compound increased the rate (generally two-fold) of reduction of the DCPIP, its use was discontinued mainly for the reasons given by Arrigoni and Singer.[21] Malic dehydrogenase activity measured as described will involve $NADH_2$-diaphorase activity, since it is the action of this enzyme which gives the "coupled" reduction of DCPIP.

[20] W. W. Umbreit, R. H. Burris and J. F. Stauffer, *Manometric Techniques*, Revised Edn., Burgess Publishing Co., Minnesota (1957).

[21] O. Arrigoni and J. P. Singer, *Nature, Lond.* 193, 1256 (1962).

Diaphorase ($NADH_2$)

The activity of this enzyme system was measured by following the decrease in absorptivity at 600 mμ, due to the reduction of DCPIP as $NADH_2$ is oxidized, in 1-cm cuvettes containing the following: 1 ml solution A (as above); 0·005–0·05 ml mitochondrial preparation, and 0·1 ml (0·15 μmole) $NADH_2$ (substrate), and water to make to 3·0 ml. Readings were taken every 30 sec for 2–3 min after the addition of substrate. The usual blank correction was made. The definitions of initial rate, enzyme units and specific activity were the same as for succinic and malic dehydrogenases.

$NADH_2$ *Cytochrome-c Reductase*

This enzyme system was determined by following the reduction of cytochrome-c by measuring the increase in absorptivity at 550 mμ in 1-cm cuvettes containing the following: 1 ml solution A (with the DCPIP solution replaced by water; in some experiments sodium azide—7·4 μmole/ml—replaced the cyanide but the results obtained were identical); 0·01–0·05 ml mitochondrial preparation; 0·1 ml (0·15 μmole) $NADH_2$ and 0·8 ml (0·09 μmole) cytochrome-c and water to make to 3·0 ml. In the control (blank) cuvette 0·1 ml of water replaced the $NADH_2$. Readings were taken every 30 sec for 2–3 min after the addition of the cytochrome-c. Initial rate, enzyme units and specific activity were the same as for diaphorase.

Succinic Cytochrome-c Reductase

This enzyme was assayed by measuring the increase in absorptivity at 550 mμ after incubating the following mixture for 10 min at 25°. One ml of solution A (minus DCPIP, as for $NADH_2$ cytochrome-c reductase); 0·02–0·05 ml mitochondrial preparations; 0·1 ml (10 μmole) sodium succinate, and water to make to 3·0 ml. The reaction was started by adding 0·8 ml (0·09 μmole) cytochrome-c after the incubation. The blanks were treated exactly the same except that 0·1 ml water replaced the succinate. Readings were taken every 30 sec but, since the activity of the enzyme fell off after about 60 sec, the initial rate was taken as the rate over the first 60 sec after the addition of the cytochrome-c. Enzyme units and specific activity were calculated as before.

Sonication of Mitochondrial Suspensions

Mitrochondrial suspensions were treated to disintegrate intact particles in an M.S.E. Ultrasonic Disintegrator (20,000 cycles; maximum output 60 W) for periods up to 40 sec. To minimize thermal destruction of enzymes the machine was located in a room at 1° and the vessel containing the suspension was surrounded by melting ice. During the period of sonication the temperature of the suspension did not rise above 4°. After sonication the suspension was centrifuged at 25,000 *g* for 15 min. The supernatant solution was used immediately for the determination of the activity of the " solubilized" dehydrogenases, while the pellet was resuspended (using the Kontes homogenizer) in the original volume of 0·2 M sucrose for the usual Warburg studies and the determination of dehydrogenase activity.

CHEMICAL ESTIMATIONS

The *phenolics content* of the mitochondrial preparations was determined by the methods of Swain and Hillis,[22] for plant extracts. We have found that sucrose interferes especially with the determination of leucoanthocyanins so that never more than 0·1 ml mitochondrial pre-

[22] T. SWAIN and W. E. HILLIS, *J. Sci. Food Agr.* **10**, 63 (1959).

paration (which is suspended in a 0·2 M sucrose medium) was taken for these estimations. It was also necessary to centrifuge off the particle debris before estimating the optical density of the final solutions. Swain (private communication) has provided calibration tables using (+)-catechin for total phenolics and flavanols, and cacao leucocyanidin for leucoanthocyanidin. Since the exact nature of the phenolic compounds present in the mitochondrial preparations is not known, the analytical results are only of value for purposes of comparison and in some preparations leucoanthocyanidin appears to be greater than total phenolics.

Nitrogen determinations were made by a method specially developed by one of us (J.D.J.) and which consisted of a combination of Kjedahl digestion followed by estimation of the ammonia formed with ninhydrin. The samples, containing 10–60 μg nitrogen, were placed in Pyrex tubes (10 mm × 100 mm, graduated at 4·0 ml) with 0·2 ml of concentrated H_2SO_4 and 0·2 ml of H_2O_2 (100 vol.) and incubated at 105° for 4 hr. A further 0·2 ml of H_2O_2 was added and the tubes transferred to close-fitting cavities (2 cm deep) in a heated aluminium block. Digestion was carried out at 140–150° until all the water was removed from the samples, and then at 300–360° for 30 min. The tubes were rapidly cooled by means of a forced draught through the heating block and 0·2 ml of H_2O_2 again added. Heating, cooling and addition of 0·2 ml of H_2O_2 were repeated until the digests remained clear at 360°. After a final cooling, 1 ml distilled water followed by 0·5 ml 10 N NaOH were added, and the contents of the tubes diluted with water to the 4-ml graduation mark and thoroughly mixed. An aliquot (0·05–0·5 ml depending on the ammonia content) was measured into Pyrex tubes 15 mm × 125 mm and the volume made to 0·5 ml with distilled water. To this 0·5 ml ninhydrin reagent[23] (with hydrindantin prepared according to Connel *et al.*[24]) was added, the solutions mixed, the tubes heated in boiling water for 15 min, cooled and the contents made to 5 ml with 50% (v/v) ethanol. The colour intensity was measured in 1-cm cells at 570 mμ. The nitrogen content was obtained from calibration curves prepared using a standard solution of ammonium sulphate treated exactly as for the sample. Such standards were also used with each batch of determination. For the determination of N in the preparations, 0·1 ml suspension was digested directly ("total N") or 0·1 ml was first extracted once with 15% trichloroacetic acid (TCA) followed by washing the centrifugate twice with 7·5% TCA followed by digestion of the final centrifugate ("TCA insol. N"). Protein = TCA insol. N × 6·25.

Paper Chromatography of Organic Acids

Separation and identification of acids present after a Warburg run were attained by removing the mitochondria by centrifugation, passing the supernatant liquid down a Dowex 50 column (H^+ form) to remove cations, concentrating the liquid leaving the column *in vacuo* in a rotary film evaporator and running the concentrate on Whatman No. 2 paper using the solvent layer of a mixture of *n*-butanol; water; formic acid (4:5:1 v/v) after equilibration overnight with the aqueous layer. The acid spots were developed by spraying with Hargreaves reagent (Hulme[25]).

Separation and Determination of Keto-acids

The method used for the extraction, separation by paper chromatography, and quantitative determination of the keto acids was essentially that of Isherwood and Niavis[26] except that

[23] S. Moore and W. H. Stein, *J. Biol. Chem.* **211**, 907 (1954).
[24] G. E. Connel, G. H. Dixon and C. S. Hanes, *Can. J. Biochem. and Physiol.* **33**, 416 (1955).
[25] A. C. Hulme. *Advances in Applied Microbiology*, Vol. 3, p. 343, Academic Press, New York (1961).
[26] F. A. Isherwood and C. A. Niavis, *Biochem. J.* **64**, 549 (1956).

solvent system (a) on paper buffered at pH 6·2 described by Isherwood and Cruickshank[27] was used. The extraction procedure was modified for mitochondrial digests as follows: to the contents of the Warburg flasks after a 2-hr run, was added 1 ml of 1% dinitrophenylhydrazine in 5 N sulphuric acid and the mixture allowed to stand at room temperature for 30 min. The contents of the flasks were then washed into centrifuge tubes with a little water. The centrifuge tubes were then spun for 5 min, the supernatant liquid containing the 2:4 dinitrophenyl hydrazones transferred to a micro-separating funnel. The solid residues in the centrifuge tubes were washed twice with ether and the washing added to the separating funnel. After separation of the other, the aqueous layer was re-extracted four times with ether, etc., as described by Isherwood and Niavis.[26]

Chemicals

Yeast concentrate was obtained from the Sigma Chemical Co. adenosine triphosphate (ATP), NAD, $NADH_2$ cytochrome-c, sodium pyruvate, and/or oxoglutarate and CoA from Boehringer u Soehne; crystalline bovine plasma albumin from Armour Pharmaceutical Co.; isocitric, oxalosuccinic and malic acids and tris from Fluka A.G.; succinic, fumaric and aconitic acids and sodium azide from Light and Co.; citric acid, nicotinamide and DCPIP from British Drug Houses. Polyvinylpyrrolidone (PVP) was a pharmaceutical grade called Kollidon 25 manufactured by the Badische Anilin u. Sodafabrik A.G. and having a molecular weight of approximately 28,000. Sulphuric acid and H_2O_2 were B.D.H. "Microanalytical" grade; ethylene glycol monomethyl ether (methyl cellosolve) B.D.H. was technical grade; ninhydrin was obtained from Koch Laboratories. All the other chemicals were of Analar grade wherever possible. Glass distilled water was used throughout.

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[27] F. A. ISHERWOOD and D. H. CRUIKSHANK, *Nature, Lond.* 173, 121 (1954).