Free Fatty Acids in Carrot-Tissue Preparations and their Effect on Isolated Carrot Mitochondria

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Mitochondria isolated from disrupted carrot tissue oxidize succinate more rapidly than all other substrates tested, have relatively high q_{O_2} values, relatively low cofactor requirements and show respiratory control with ADP only when bovine serum albumin (1%) is present in the homogenization medium. A protective effect of albumin during the isolation or incubation of mitochondria from insect (Lewis & Slater, 1954; Sacktor, O'Neill & Cochran, 1958) and mammalian (Polis & Shmukler, 1957; Weinbach, 1959; Helinski & Cooper, 1960) sources also occurs, and is probably due to its ability to bind free fatty acids (Boyer, Ballou & Luck, 1947; Goodman, 1958). No similar data are available on the mode of action of albumin during the preparation of plant mitochondria. Accordingly the production of free fatty acids during the preparation of carrot mitochondria and the effect of these fatty acids on the function of isolated mitochondria have been investigated. It is concluded that the presence of a variety of fatty acids during the isolation of carrot mitochondria could account for the differences observed in mitochondria prepared with and without added albumin in the homogenization medium.

MATERIALS AND METHODS

Materials

Apart from those specified below, organic acids, amino acids, coenzymes and other chemicals used were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Bovine serum albumin (Cohn fraction V) was from Commonwealth Serum Laboratories, Melbourne, Australia. *N*-Acetyl-*N*-methylurea was from British Drug Houses Ltd. *L*-Thyroxine (sodium salt, pentahydrate) was from Glaxo Laboratories, Greenford, Middlesex. All fatty acids used were from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. Fatty acids were of the highest grade available and unsaturated acids were stored at -15° under nitrogen; salts were formed by adjusting the pH to 8 with NaOH. EDTA (disodium salt), potassium dihydrogen phosphate, disodium hydrogen phosphate and sucrose were analytical reagents. The disodium salt of ATP and the sodium salt of AMP were used. Alcohol dehydrogenase was prepared by the method of Racker (1950).

Determinations

Fatty acids were determined, as methyl esters, after the removal of the light petroleum solvent, by the method of Stern & Shapiro (1953).

Phosphate was determined by the method of Allen (1940).

Protein was determined by the method of Cleland & Slater (1953) as modified by Dalgarno & Birt (1962a).

Preparation of carrot mitochondria

Carrot mitochondria were prepared by the method of Dalgarno & Birt (1962a); where used, serum albumin (1%) was present only in the homogenization medium. Frequently, particles giving high P:O ratios and q_{O_2} values with succinate and malate could not be isolated from carrots harvested in Victoria in July and August (see the Results section).

Extraction of lipids

Homogenates. For the extraction of free fatty acids from homogenates, diced carrot tissue was homogenized in two batches each of 150 g. with sucrose-phosphate buffer medium (160 ml.) with or without 1% (w/v) serum albumin (for details see Dalgarno & Birt, 1962a); each homogenate (200 ml.) was then squeezed through two layers of muslin. The two preparations (each representing 150 g. of tissue) were then kept at 4° for 25 min. or 60 min., rapidly frozen in liquid air and subsequently freeze-dried for about 20 hr. The freeze-dried preparation was extracted three times with vigorous stirring in chloroform-ethanol (2:1, v/v); the extract was filtered on Whatman no. 1 filter paper and the filtrate evaporated to dryness by warming under reduced pressure. The residue was dissolved in diethyl ether, the extract washed three times with 0.1N-hydrochloric acid and once with water (to remove non-lipid impurities), and then evaporated to dryness under reduced pressure. The material was then ready for methylation.

Mitochondria. Two samples, each of 150 g. of tissue, were used for the preparation of mitochondria as described by Dalgarno & Birt (1962a); the final suspension of particles (5 ml.) was immediately frozen in liquid air and freeze-dried for 20 hr., and the solid extracted as above.

Albumin and supernatant fluid. In experiments where fatty acids present in the precipitated albumin and supernatant fractions were determined, albumin was removed from the homogenate with ammonium sulphate (Dalgarno & Birt, 1963); both the albumin and the protein-free supernatant fluid were freeze-dried and the solids extracted as described above. Fatty acids already present in the albumin were extracted by treating 5 g. of albumin at

full speed for 2 min. in a Waring Blendor with 200 ml. of chloroform-ethanol; the residue was filtered on Whatman no. 1 filter paper, and the filtrate was evaporated to dryness, the solid extracted with chloroform and the extract again evaporated to dryness. This procedure was more drastic than that used for the extraction of free fatty acids from homogenates and may be responsible for a disparity observed in the concentrations of free fatty acids in these two types of preparation.

Total tissue lipid. Lipid was extracted by homogenizing peeled diced carrot tissue (about 40 g.) for 2 min. in a Waring Blendor containing approx. 300 ml. of chloroform-ethanol (2:1, v/v). The supernatant fluid was filtered through Whatman no. 1 filter paper and the residue extracted twice with chloroform-ethanol. The three extracts were combined and evaporated to dryness under reduced pressure; the residue was extracted with chloroform and the solvent evaporated. The lipid was then ready for methylation.

Mitochondrial lipid. Mitochondria were prepared from 150 g. of tissue in an homogenization medium containing 1% (w/v) of serum albumin (see Dalgarno & Birt, 1962a). The pellet was extracted with 50 ml. of chloroform-ethanol, the supernatant fluid separated by filtration and the residue re-extracted with chloroform-ethanol as above. The solvent was evaporated from the combined supernatant fluids, the residue extracted with chloroform and the extract again evaporated to dryness.

Preparation of methyl esters

Free fatty acids. Freshly prepared diazomethane in diethyl ether was added to lipid samples; after 30 min. in the dark the diazomethane and diethyl ether were removed by evaporation under reduced pressure. The methyl esters of free fatty acids were freed from non-volatile contaminants by sublimation (0.05 mm. Hg) on to a cold-finger (see Stoffel, Chu & Ahrens, 1959) and dissolved in 100 ml. of light petroleum (b.p. 40–70°); 5 ml. samples were taken for quantitative analysis, and the light petroleum was removed from the remainder by gentle warming under a stream of nitrogen. The residue was used for gas-chromatographic analysis.

The procedure used for the extraction of free fatty acids from homogenates, unlike that used for the preparation of a total tissue-fatty acid sample, yielded methyl esters containing impurities that appeared during gas chromatography as a 'hump' between the air peak and methyl palmitate or methyl stearate. This material could be removed before methylation of the fatty acids by sublimation on to a cold-finger, but, as even non-methylated fatty acids sublimed under these conditions, this procedure was not adopted as a routine measure for removing the contaminating material.

Total fatty acids. The sample of tissue or mitochondrial lipid was refluxed for 3 hr. in methanolic 1.5N-hydrochloric acid and sublimed on to a cold-finger by the method of Stoffel *et al.* (1959).

Gas chromatography

The apparatus on which all methyl esters were analysed employed a glass column (6 ft. or 12 ft. long, 4 mm. internal diameter) packed with Gas-Chrom P (80–100 mesh) coated with 13% poly(ethylene glycol adipate) (Applied Science

Laboratories Inc., State College, Pa., U.S.A.). The column was operated at 175° or 197° with an argon flow-rate of about 40 ml./min. and an ionization detector, and was calibrated with a standard mixture of pure fatty acid methyl esters, supplied by National Institutes of Health, Bethesda, Md., U.S.A. For some samples a Pye Argon chromatograph was used with a straight stainless-steel column (4 ft. long, 5 mm. internal diameter), packed with Gas-Chrom P (80–100 mesh) and coated with 25% poly(ethylene glycol adipate) (LAC-2-R 446; Cambridge Instrument Co. Inc., Cambridge, Mass., U.S.A.) and with 2% phosphoric acid. The column was operated at 205°; the injection temperature was 300°.

Methyl esters were identified by comparison of their retention times with those of known mixtures. Some other peaks were tentatively identified by comparison of their retention times, relative to methyl stearate, with published results (Farquhar, Insull, Rosen, Stoffel & Ahrens, 1959). The approximate position of unidentified peaks is noted in the appropriate Tables, together with their percentage by weight. A molar percentage of the unknown component A (see Tables 1–3) was calculated by assuming it to be a saturated C_{11} fatty acid.

Fatty acids bound to added albumin. The molar percentages of the major fatty acids already associated with the albumin added to the homogenization medium were: peak A, 40%; myristic acid, 1%; palmitic acid, 8%; palmitoleic acid, 4%; heptadecanoic acid, 0.5%; stearic acid, 4%; oleic acid, 36%; linoleic acid, 5%; linolenic acid, 1.5%. Approximately 5% by weight of the total fatty acids were unidentified.

Determination of enzymic activities

A Beckmann DK-2 ratio recording spectrophotometer was used for the determinations, which were performed at constant temperature (20°). All reaction mixtures contained either 50 or 100 μ l. of the mitochondrial preparation, depending on the resulting turbidity; 50 μ l. contained approx. 0.5 mg. of protein (including any adsorbed albumin). Cells of 1 cm. light-path were used and the total volume in the cell was 3 ml. Small volumes (less than 0.1 ml.) and NAD, NADH₂ or 2,6-dichlorophenol-indophenol were added finally for the determination of malate-dehydrogenase, dihydronicotinamide-adenine dinucleotide-dehydrogenase and succinate-dehydrogenase activities respectively; the contents of the cell were rapidly stirred and readings commenced. The activities of dihydronicotinamide-adenine dinucleotide dehydrogenase and succinate dehydrogenase are based on extinction changes occurring in the first 2½ min. after the final addition, and for malate dehydrogenase, which rapidly reaches equilibrium, in the first 30 sec. The activities were determined as follows.

Succinate-dehydrogenase activity. This was determined from the decrease in extinction at 600 m μ after the addition of 2,6-dichlorophenol-indophenol (final concn. 0.017 mM) to potassium cyanide (1 mM), phenazine methosulphate (1 mg./ml.), succinate (50 mM), phosphate buffer (sodium and potassium salts), pH 7.1 (10 mM), and mitochondria. Both potassium cyanide and phenazine methosulphate increased the rate of reduction of 2,6-dichlorophenol-indophenol by about 20%. Activity was about 60% greater when determined in a medium of low osmolarity (no sucrose present apart from that added with the mito-

chondrial preparation) rather than in a medium of 0.3M-sucrose. All determinations were performed in the medium of low osmolality.

Malate-dehydrogenase activity. This was determined from the increase in extinction at 340 m μ after the addition of NAD (final concn. 1 mM) to a solution containing malate (50 mM), phosphate buffer (sodium and potassium salts), pH 7.1 (10 mM), and mitochondria. With freshly prepared particles, the rate of reduction during the first 30 sec. was 4 times as great in a reaction medium of low osmolality as in a medium of 0.3M-sucrose. All determinations were performed in the medium of low osmolality.

Dihyronicotinamide-adenine dinucleotide-dehydrogenase activity. This was determined from the decrease in extinction at 340 m μ after the addition of NADH₂ (final concn. 0.06 mM) to a solution containing phosphate buffer (sodium and potassium salts), pH 7.1 (10 mM), and mitochondria in a medium of low osmolality (see above).

Measurement of oxygen consumption

The conventional Warburg apparatus was used. Mitochondrial q_{O_2} values have not been corrected for albumin adsorbed on the pellet.

Determination of phosphate esterification

Substrates and inhibitors were added from different side arms after incubation of the mitochondrial suspension at 30° for 10 min. The reaction mixture consisted of sucrose (0.3M), magnesium chloride (5 mM), AMP (1 mM), ATP (1 mM), NAD (1 mM), EDTA (1 mM), phosphate buffer (sodium and potassium salts), pH 7.1 (16 mM), freshly prepared hexokinase (2 mg./flask, 300 Kunitz-McDonald units) and glucose (86 mM) in a total volume of 2.5 ml. Except in the experiment described in Table 5, fatty acids were warmed and added to the incubation medium just before the addition of the glucose-hexokinase solution and the mitochondria; the latter had therefore been preincubated with the fatty acid for approx. 15 min. before readings were commenced. A sample for the determination of the initial phosphate concentration was taken from the control flask immediately after the addition of substrates. Incubation with different substrates, uncouplers of oxidative phosphorylation or fatty acids was continued until approx. 7 μ g. atoms of oxygen had been taken up; where oxygen uptake was slow the sample for phosphate analysis was taken after approx. 1 hr. (see the Tables). Some phosphate esterification was observed before the addition of substrate, but only with mitochondria prepared in the presence of albumin.

Although albumin was not present in the medium used for the final resuspension of the mitochondria, it is probable that some of this protein was adsorbed on the particles during the disruption of the tissue (see Dalgarno & Birt, 1962a); however, in all the experiments reported, the amounts of albumin transferred in this way to the incubation vessels could not have decreased the concentrations of the fatty acids added as uncouplers of oxidative phosphorylation and respiratory inhibitors by more than 2%.

RESULTS

Free fatty acids in carrot preparations obtained by homogenizing without added albumin. The concen-

tration of free fatty acids in an homogenate, prepared by blending carrot tissue in a sucrose-phosphate buffer medium without added albumin and kept at 4° for 25 min., was 0.013 mM; after 60 min. the concentration rose to 0.041 mM. The concentration of free fatty acids in the mitochondrial suspension obtained from this homogenate was 1.1 mM. Table 1 shows the proportions of free fatty acids in these three fractions. Comparison of the homogenates shows that between 25 min. and 60 min. there was a 16-fold increase in the proportion of a component that was rapidly eluted on gas chromatography; this represented a 50-fold increase in the total amount of the component. From its relative retention time it could have been a saturated C₁₁ fatty acid, the iso-form of a saturated C₁₂ acid or an unsaturated C₁₀ isoprenoid derivative. The free fatty acids in the mitochondrial fraction are predominantly palmitic acid and a C₁₈ monounsaturated acid. The existence of petroselenic acid in quantity in the seed fat of *Umbelliferae* (e.g. 58% in carrot-seed fat; Hilditch, 1941) suggests that not all the C₁₈ monoenoic acid observed was oleic acid.

Free fatty acids in carrot preparations obtained by homogenizing with added albumin. The concentration of free fatty acids in an homogenate, prepared by blending carrot tissue in a sucrose-phosphate medium containing serum albumin (1%) and kept at 4° for 25 min., was 0.015 mM; after 60 min. the concentration rose to 0.11 mM (determined by summing the amounts present in all fractions). The concentration of fatty acid in the mitochondrial suspension was 0.56 mM, i.e. about half that obtained in the suspension prepared without added albumin. Fatty acids associated with the albumin precipitated from this homogenate after 60 min. accounted for 81% of the total fatty acids in the homogenate (21.6 μ moles); the mitochondrial pellet contained 13%, and the supernatant liquid remaining after removal of albumin 6%, of the total free fatty acids.

The albumin used in these experiments contained adsorbed fatty acids (approx. 3 μ moles of fatty acid/g. of albumin); consequently determinations of the amounts of free fatty acids released from tissue disrupted in a medium containing albumin should be corrected for this contamination. Each homogenate (200 ml.) contained 1.6 g. of albumin, i.e. sufficient to introduce about 4.8 μ moles of fatty acid. Thus, in the experiment described in Table 2, it is apparent that there has been a negligible release of fatty acids from tissue lipids in the first 25 min. after homogenizing. However, in a comparable experiment without added albumin in the homogenization medium (Table 1), 2.6 μ moles of fatty acid were detected in the homogenate after 25 min. Therefore a release of

fatty acids from the tissue does occur in the absence of added albumin.

Of the total free fatty acids associated with the precipitated albumin, 84 % consisted of the peak A component, palmitic acid, stearic acid and a C_{18} monoenoic acid (Table 2). The same fatty acids predominated in the supernatant fluid, except for the peak A component. Although the peak A compound was bound to the added albumin, it was also present in the tissue itself, since it appeared in relatively large amounts in homogenates prepared without added albumin and kept at 4° for 60 min. (Table 1).

The mitochondrial suspension contained a higher proportion of unsaturated free fatty acids (62 %) than did the precipitated albumin (44 %); however, mitochondria prepared without added albumin contained a smaller percentage of unsaturated free fatty acids than did the homogenates aged either for 25 min. or for 60 min. (Table 1), if it is assumed that peak A represents a saturated fatty acid. Of the free fatty acids in the mitochondrial fraction prepared with added albumin, 79 % were accounted for by palmitic acid, the C_{18} monoenoic acid, the C_{18} dienoic acids and linolenic acid.

Total fatty acids of tissue and mitochondrial lipid. The proportions of free fatty acids in the various preparations described were compared with those of the total esterified fatty acids of the tissue. About 0.3 % of the fresh weight of the tissue was extractable and the proportions of fatty acids in homogenates and mitochondrial preparations differed substantially from the total fatty acid content of the tissue or the mitochondrial lipids. The esterified fatty acids of the tissue were predominantly linoleic acid, palmitic acid and linolenic acid; stearic acid and oleic acid contributed up to 5 % and up to 14 % respectively of the total (Table 3).

The fatty acids of the mitochondrial fraction also contained large amounts of linoleic acid, palmitic acid and linolenic acid; in addition a C_{18} dienoic acid and an unknown acid (R_f 2.81) were detected in large amounts in the mitochondrial lipid. In the two analyses shown in Table 3, these two components accounted for 40 % of the mitochondrial fatty acids, but did not occur in sufficient amount in the total lipid of the tissue to be detectable.

Effect of homogenizing carrot tissue with and without added albumin on the phosphorus:oxygen

Table 1. *Free fatty acids in homogenates and mitochondria prepared without added albumin*

The results are based on two sets of determinations. The homogenate aged for 25 min., the homogenate aged for 60 min. and the mitochondrial suspension contained 2.6, 8.2 and 5.5 μ moles of free fatty acid respectively. Each of the two homogenates was prepared from 150 g. of tissue and the mitochondrial suspension (5 ml.) from 300 g. of tissue.

Fatty acid	Proportion of methyl ester in measurable peaks (moles %)		
	Homogenate aged for 25 min.	Homogenate aged for 60 min.	Mitochondria from homogenate aged for 60 min.
Peak A	3	49	22
Lauric acid	15	3	2
Myristic acid	2	2	4
Isomyristic acid	12	2	4
Pentadecanoic acid	1	1	1
Palmitic acid	3	13	19
Palmitoleic acid	8	4	5
Heptadecanoic acid	1	—	—
Stearic acid	11	8	8
Oleic acid	18	7	17
Linoleic acid	5	3	5
Octadeca-6,9-dienoic acid	1	1	2
Linolenic acid	18	5	5
Arachidic acid	4	2	6
Position	Proportion of unidentified methyl esters (% by weight)		
Between peak A and lauric acid	1	—	1
Between lauric acid and myristic acid	1	2.5 (2 peaks)	8 (3 peaks)
Between pentadecanoic acid and palmitic acid	—	1	2
Between heptadecanoic acid and stearic acid	2	—	—
After arachidic acid	4 (2 peaks)	—	6.5 (2 peaks)

Table 2. *Free fatty acids in tissue preparations obtained with albumin in the homogenization medium*

The results are based on two sets of determinations. The homogenate aged for 25 min., and the precipitated albumin, supernatant fluid and mitochondrial suspension (all from 60 min. homogenates aged for 60 min.), contained 3.0, 17.5, 1.3 and 2.8 μ moles of free fatty acid respectively. The homogenates were each prepared from 150 g. of tissue and the mitochondrial suspension (5 ml.) was from 300 g. of tissue.

Fatty acid	Proportion of methyl ester in measurable peaks (moles %)			
	Homogenate aged for 25 min.	Precipitated albumin from homogenate aged for 60 min.	Supernatant from homogenate aged for 60 min.	Mitochondria from homogenate aged for 60 min.
Peak A	—	31	—	Trace
Lauric acid	—	—	6	—
Myristic acid	8	2	7	4
Isomyristic acid	—	1	—	—
Pentadecanoic acid	—	1	8	—
Palmitic acid	18	13	21	21
Palmitoleic acid	—	3	—	4
Heptadecanoic acid	—	1	—	—
Stearic acid	30	7	18	7
Oleic acid	44	33	40	25
Linoleic acid	—	6	—	15
Octadeca-6,9-dienoic acid	—	1	—	9
Linolenic acid	—	1	—	9
Position	Proportion of unidentified methyl esters (% by weight)			
	Between lauric acid and myristic acid	Between palmitoleic acid and stearic acid	Between heptadecanoic acid and stearic acid	After arachidic acid
	—	0.2	—	—
	—	—	—	4
	—	0.7	—	—
	—	—	—	8

Table 3. *Total fatty acids, free and combined, of tissue lipid and mitochondrial lipid*

Experimental details are given in the text.

Fatty acid	Proportion of methyl ester in measurable peaks (moles %)			
	Total fatty acids of tissue		Mitochondrial fatty acids	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Peak A	0.1	0.1	0.2	0.8
Lauric acid	—	—	0.3	—
Isomyristic acid	—	—	4.8	—
Myristic acid	2.2	0.7	0.6	0.2
Pentadecanoic acid	0.2	0.6	0.8	0.8
Palmitic acid	21.6	27.4	26.1	31.0
Palmitoleic acid	—	1.1	0.7	0.8
Heptadecanoic acid	2.7	0.2	0.3	0.6
Stearic acid	0.5	5.2	1.5	1.4
Oleic acid	1.7	13.7	2.5	3.8
Linoleic acid	63	47.5	59	55
Linolenic acid	7.5	3.5	3.1	5.8
Eicosa-11,14-dienoic acid	0.2	—	—	—
Arachidonic acid	0.5	—	—	—
Position	Proportion of unidentified methyl esters (% by weight)			
	Between heptadecanoic acid and stearic acid	Between linoleic acid and linolenic acid	After eicosa-11,14-dienoic acid	
	—	—	—	7.0
	—	—	2.2	(R_T 0.82)
	—	—	(R_T 1.58)	26.0
	0.8	—	37	(R_T 1.58)
			(R_T 2.81)	14
				(R_T 2.81)

Table 4. *Effect of homogenizing carrot tissue with and without added albumin on the q_{O_2} values and phosphorus: oxygen ratios of mitochondria*

The concentration of each substrate was 20 mM, except for that of $NADH_2$ which was 5 mM. All flasks except the one to which $NADH_2$ was added also contained NAD (1 mM). There was approx. 7 mg. of protein/flask for mitochondria prepared with and without albumin; the q_{O_2} values have not been corrected for albumin adsorbed on the mitochondria. The incubation temperature was 25°.

Substrate	Homogenized without added albumin			Homogenized with albumin		
	q_{O_2}	P:O ratio	Duration of phosphate uptake (min.)	q_{O_2}	P:O ratio	Duration of phosphate uptake (min.)
Succinate	6.3	0.0	60	43.5	1.6	20
Malate	26.4	0.1	30	39.9	1.9	25
Citrate	7.5	0.0	60	17.5	2.0	35
α -Oxoglutarate	1.0	0.0	60	10.8	2.0	30
$NADH_2$	21.0	0.05	30	44.4	1.3	20

ratios of isolated mitochondria. Carrot mitochondria prepared by homogenizing with added serum albumin gave P:O ratios of 1.6, 1.9, 2.0, 2.0 and 1.3 respectively with succinate, malate, citrate, α -oxoglutarate and $NADH_2$ as substrates (Table 4). Mitochondria isolated from the same batch of tissue without added albumin showed either complete or almost complete lack of phosphorylation (P:O values less than 0.1). With succinate, malate, citrate, α -oxoglutarate and $NADH_2$, mitochondria prepared without added albumin gave q_{O_2} values that were only 15, 64, 43, 9 and 47 % respectively of the corresponding values for mitochondria prepared with added albumin. The ratio of the oxygen consumption with succinate to that with malate for mitochondria prepared with and without added albumin is 1.09 and 0.24 respectively (cf. Dalgarno & Birt, 1962*a, b*). The calculation of these q_{O_2} values does not include a correction factor for protein in the mitochondrial fraction which was derived from the albumin; the q_{O_2} values given for mitochondria prepared with added albumin are therefore probably too low.

Effect of various compounds on the phosphorus: oxygen ratios obtained with carrot mitochondria prepared with added albumin. The effect on P:O ratios and q_{O_2} values of various compounds known to uncouple oxidative phosphorylation in animal mitochondria has been tested with malate and succinate as substrates (Table 5). Oxygen uptake with malate was not substantially affected by either 2,4-dinitrophenol (0.1 mM), oleate (0.001 mM), thyroxine (0.05 mM) or calcium chloride (1 mM); 2,4-dinitrophenol and oleate decreased the P:O ratios with malate by 79 % and 29 % respectively. Oxygen uptake with α -oxoglutarate was inhibited 27 % and 22 % by 2,4-dinitrophenol and oleate respectively, stimulated slightly by calcium chloride (25 %) and was virtually unaffected by thyroxine. Phosphorylation with α -oxoglutarate

Table 5. *Effect of uncoupling agents on the phosphorus: oxygen ratios of carrot mitochondria prepared with added albumin*

The concentration of each substrate was 40 mM, that of thyroxine was 0.05 mM, that of oleate was 0.001 mM; that of 2,4-dinitrophenol was 0.1 mM and that of $CaCl_2$ was 1 mM. Phosphate uptake was continued for 30 and 40 min. with malate and α -oxoglutarate respectively. There was approx. 4 mg. of protein/flask. The q_{O_2} values are not corrected for albumin adsorbed on the mitochondria. The incubation temperature was 25°.

Additions	Malate		α -Oxoglutarate	
	q_{O_2}	P:O ratio	q_{O_2}	P:O ratio
None	42	1.4	23	1.8
Dinitrophenol	41	0.3	17	0.1
Oleate	43	1.0	18	1.8
Thyroxine	40	1.4	25	2.1
$CaCl_2$	39	1.2	29	1.7

was almost completely inhibited by 2,4-dinitrophenol but was not inhibited by oleate at this concentration.

Effect of oleate concentration on the oxidation of malate and succinate by carrot mitochondria prepared with added albumin. As oleate was present in the carrot homogenates in relatively high concentrations and since there are a number of reports of its effect on various properties of animal mitochondria (see the Discussion section), it was added at three different concentrations to carrot mitochondria oxidizing succinate and malate (Table 6).

With 0.1, 1 and 10 mM-oleate, oxygen uptake with succinate was inhibited to a greater extent than with malate; the differential effect was most marked with 1 mM-oleate (malate oxidation, not inhibited; succinate oxidation, inhibited by 79 %). Phosphorylation with both substrates was little affected by 0.1 mM-oleate, but was completely

prevented when the concentration of the fatty acid was 1 mm or greater.

Qualitatively similar results were obtained with the same concentrations of stearate, though the relative effectiveness of stearate and oleate varied somewhat in different experiments. It is possible that differences in the physical properties of the fatty acid preparations used in different experiments were responsible for this variability (see the Discussion section).

Effect of various long-chain fatty acids on the q_{O_2} values and phosphorus:oxygen ratios of carrot mitochondria oxidizing succinate and malate. Table 7 shows the effect of a range of fatty acids (all 1 mm) on the q_{O_2} values and P:O ratios of carrot mitochondria prepared with added albumin and oxidizing succinate or malate. With succinate as substrate all fatty acids tested, except butyrate, inhibited oxygen uptake. The greater inhibitions were induced by laurate (89%), oleate (88%), linolenate (83%), myristate (81%) and linoleate (78%); less inhibition was observed with palmitate (57%), stearate (32%) and arachidate (17%). The fatty acids most effective in inhibiting oxygen

uptake were generally most effective in lowering P:O ratios; thus laurate and linolenate completely inhibited phosphorylation, and myristate, palmitate, linoleate, oleate and stearate lowered P:O ratios to 3, 12, 12, 17 and 52% respectively of the control value without fatty acid. Neither butyrate nor arachidate lowered the P:O ratio below that of the control.

In an experiment with malate as substrate, the extent of inhibition exerted by the various fatty acids was generally similar to that obtained with succinate (Table 7). There was some evidence for a greater inhibition of oxygen uptake with succinate than with malate (cf. results for both substrates with stearate, oleate and arachidate, Table 7), but it was not as marked as that reported in Table 6 (cf. malate and succinate oxidation with 1 mm-oleate). The differences between the results in Tables 6 and 7 re-emphasize the variability in response to fatty acids especially at about 1 mm concentration.

During some stages in the growing season of the carrot, mitochondria with high (greater than 2) P:O ratios with malate could not be isolated (see

Table 6. *Effect of oleate on the oxygen uptake and phosphorus:oxygen ratios of carrot mitochondria oxidizing succinate and malate*

The mitochondria were prepared with added albumin; the concentration of each substrate was 40 mM. There was approx. 4 mg. of protein/flask. The q_{O_2} values are not corrected for albumin adsorbed on the mitochondria. The incubation temperature was 25°.

Concn. of oleate (mM)	Duration of phosphate uptake (min.)	Succinate		Malate	
		q_{O_2}	P:O ratio	q_{O_2}	P:O ratio
0.0	20	81	1.47	69	2.22
0.1	20	71	1.65	64	2.0
1	35	17	0.0	70	0.0
10	35	12	0.0	16	0.0

Table 7. *Effect of fatty acids on the q_{O_2} values and phosphorus:oxygen ratios of carrot mitochondria oxidizing succinate and malate*

The concentration of each substrate was 40 mM, and the final concentration of each added fatty acid was 1 mM. The incubation temperature was 30°. In the experiments with succinate and malate there was approx. 13 and 5 mg. of protein/flask respectively. All other conditions are as in Table 4.

Fatty acid	Succinate			Malate		
	q_{O_2}	P:O ratio	Duration of phosphate uptake (min.)	q_{O_2}	P:O ratio	Duration of phosphate uptake (min.)
None	32	1.53	20	42	0.85	30
Butyric acid	31	1.71	20	43	0.84	30
Lauric acid	4	0.0	55	7	0.09	60
Myristic acid	6	0.04	55	8	0.08	60
Palmitic acid	13	0.18	30	16	0.04	30
Stearic acid	22	0.79	25	38	0.42	30
Oleic acid	4	0.26	55	9	0.0	60
Linoleic acid	7	0.18	55	10	0.13	60
Linolenic acid	5	0.0	55	8	0.0	60
Arachidic acid	26	1.6	20	44	0.79	30

Table 8. *Effect of oleate and stearate on malate-dehydrogenase, succinate-dehydrogenase and dihydronicotinamide-adenine dinucleotide-dehydrogenase activities of carrot mitochondrial preparations*

The final concentration of succinate and malate was 50 mM, that of NADH₂ was 0.06 mM, and that of stearate and oleate (sodium salts) was 1 mM. Activities are based on initial rates (see the Materials and Methods section). Other conditions are as described in the Materials and Methods section. Mitochondria were prepared with albumin (1%) in the homogenization medium.

	Age of preparation (hr. at 4°)	Additions	Activity (change in E/hr.)
Malate dehydrogenase	5	Malate	6.6
	5	Stearate	2.4
	5	Malate + stearate	9.6
	5	Oleate	0.0
	5	Malate + oleate	7.2
Succinate dehydrogenase	1	Succinate	3.1
	1	Succinate + stearate	1.9
	1	Succinate + oleate	1.9
	5	Succinate	1.9
	5	Succinate + stearate	1.9
	5	Succinate + oleate	1.7
Dihydronicotinamide-adenine dinucleotide dehydrogenase	1	NADH ₂	3.1
	1	NADH ₂ + stearate	0.48
	1	NADH ₂ + oleate	0.24

the Materials and Methods section); the low P:O ratio (0.85) observed with malate alone (Table 7) is an example of this difficulty. However, the pattern of inhibition of phosphorylation by the different fatty acids was similar with both malate and succinate. With both substrates phosphorylation was more readily inhibited by a particular fatty acid than was oxygen uptake (e.g. palmitate lowered oxygen uptake and the P:O ratio with malate by 21 and 95% respectively); this effect was generally more marked with malate than with succinate.

Effect of various short-chain fatty acids on the q_{O_2} values and phosphorus:oxygen ratios of carrot mitochondria oxidizing succinate. The effect on q_{O_2} values and P:O ratios with succinate of a variety of short-chain fatty acids was tested by incubating mitochondria, prepared with added albumin (1%), with acetate, *n*-butyrate, isobutyrate, isovalerate, hexanoate, octanoate and laurate (all 1 mM). The control P:O ratio was 0.86 and with the various fatty acids P:O ratios were 0.81, 0.69, 0.82, 0.77, 0.52, 1.1 and 0.0 respectively. With all fatty acids except laurate q_{O_2} values were lowered by between 16 and 30%; with laurate the inhibition was 93%. Thus the ability of fatty acids to inhibit phosphorylation and oxygen uptake suddenly increases with a change in chain length from C₈ to C₁₂; previous results suggest that the inhibitory effect was maximal with the C₁₂ acid.

Inhibition of malate dehydrogenase, succinate dehydrogenase and dihydronicotinamide-adenine dinucleotide dehydrogenase by stearate and oleate. The difference in extent to which stearate and oleate inhibited the oxygen uptake of mitochondria

oxidizing succinate and malate may have been due to different sensitivities to the fatty acids at the level either of the electron-transport chain or of the dehydrogenase. Therefore the effect of stearate and oleate on malate-dehydrogenase, succinate-dehydrogenase and dihydronicotinamide-adenine dinucleotide-dehydrogenase activities of carrot mitochondria prepared with added albumin was investigated.

(1) *Malate dehydrogenase.* No inhibition of malate-dehydrogenase activity (determined by the reduction of NAD) was observed with 1 mM-stearate or 1 mM-oleate (Table 8). Stearate, but not oleate, slowly reduced NAD added to a preparation; the increase in extinction at 340 m μ was due to the formation of NADH₂, since the addition of acetaldehyde and alcohol dehydrogenase resulted in a fall in extinction to the initial value.

(2) *Succinate dehydrogenase.* Succinate-dehydrogenase activity was substantially inhibited (39%) by both stearate and oleate (Table 8); the extent of inhibition depended on the age of the preparation, and was apparently greater with oleate than with stearate. This latter effect may have been due to the reduction of 2,6-dichlorophenol-indophenol by stearate (see above) but this possibility was not investigated. Stearate (1 mM) lowered the high initial succinate-dehydrogenase activity in particles aged for 60 min. by 19%, but did not affect the lower activity of particles aged for 300 min.; oleate (1 mM) lowered the activity of the two preparations by 50% and 12% respectively. In another experiment with particles isolated by homogenizing in a medium containing albumin, succinate-dehydrogenase activity was 3.6, 2.9, 2.6

and 1.7 units (change in extinction/hr.; see Table 8) in particles aged without added albumin for 60, 120, 180 and 300 min. respectively; the decrease in activity after 300 min. was partially prevented (2.4 units instead of 1.7 units) if aging was carried out in a medium containing albumin (2 mg./ml.). (3) Dihydropyridine-adenine dinucleotide dehydrogenase. Dihydropyridine-adenine dinucleotide-dehydrogenase activity (determined from the rate of oxidation of 0.06 mM-NADH₂) was lowered to 15% and 8% of the control value by 1 mM-stearate and 1 mM-oleate respectively (Table 8).

Thus both stearate and oleate strongly inhibited at least two enzymes that are structurally bound to the mitochondrial-membrane system (Green & Fleischer, 1960), but did not inhibit malate dehydrogenase, a mitochondrial enzyme that is not structurally bound to the membrane system (Bendall & de Duve, 1960).

DISCUSSION

Role of serum albumin in preserving the metabolic functions of mitochondria. The problem of whether plant mitochondria can be prepared which are 'intact' by some of the criteria normally applied to animal mitochondria (respiratory control with ADP, lack of oxidation of 'external' NADH₂) has recently aroused considerable interest (see Dalgarno & Birt, 1962a, b; Bonner & Voss, 1961; Hackett, Rice & Schmid, 1960). The results of Dalgarno & Birt (1962a, b) and those in the present paper indicate that the inclusion of 1% of bovine serum albumin in the homogenization medium permits the isolation of mitochondria from carrot tissue which exhibit relatively high q_{O_2} values and P:O ratios with a variety of substrates, respiratory control with ADP and a comparatively low requirement for added cofactors. Further, with mitochondria prepared with added albumin, the ratio of oxygen uptake with malate to that with succinate more closely resembles that found in animal preparations (cf. Lardy & Wellman, 1952; Bellamy & Bartley, 1960).

Although a direct lubricating effect of the added albumin during the homogenizing may help to preserve mitochondrial structure, the results obtained suggest that the prime effect of serum albumin is to lower the concentration of mobile inhibitory fatty acids in the homogenate and the mitochondrial suspension; the concentration of free fatty acids in a tissue preparation will depend on the balance between the rate of formation of the fatty acid by hydrolysis of lipid and the rate of removal by oxidation or by re-incorporation into lipid (for data on the latter possibility, see Wojtczak & Lehninger, 1961). Fatty acids may be released in

homogenates of plant tissues by the action of a relatively non-specific lipase (e.g. that in wheat germ; Singer & Hofstee, 1948) in combination with the phospholipases commonly found in plant tissue (phospholipase C in barley and spinach chloroplasts, and phospholipase D in carrot and cabbage tissue; Dawson, 1962). Reports of fatty acid oxidation by plant mitochondria in tissues that are capable of rapidly metabolizing fat (see Stumpf & Barber, 1956; Stumpf & Bradbeer, 1959), and the information available for carrot mitochondria (Dalgarno & Birt, 1962b; and unpublished work), indicate that the oxidation of fatty acids by plant mitochondria proceeds very slowly. These considerations may explain the invariable accumulation of inhibitory concentrations of fatty acids during the disruption of carrot tissue and the preparation of mitochondria.

Previous results (Dalgarno & Birt, 1962b) have shown that the addition of albumin during the incubation of carrot mitochondria, prepared in a medium without added albumin, is ineffective in permitting phosphorylation (cf. Pierpoint, 1960); with other tissues such an absolute requirement for albumin in the homogenization medium is not shown. Thus, with flight-muscle particles from insects, the inclusion of albumin in the incubation medium alone was adequate to preserve phosphorylation (Lewis & Slater, 1954; Sacktor *et al.* 1958); without albumin the P:O ratios were decreased substantially; such particles are almost incapable of oxidizing fatty acids (see Gilmour, 1961; Sacktor, 1955; cf. Meyer, Preiss & Bauer, 1960). Mammalian mitochondria, which readily oxidize fatty acids (Grafflin & Green, 1948; Kennedy & Lehninger, 1949), can give high P:O ratios without the addition of albumin to either the homogenization or incubation media. However, a decline in phosphorylating ability during incubation has been observed with rat-liver mitochondria, and this is retarded by resuspension of the mitochondria in fresh medium (Roodyn & Suttie, 1961); this procedure may remove fatty acids generated by the particles themselves. In addition, Emmelot & Bos (1961) have shown a correlation between the ability of rat-hepatoma mitochondria to oxidize hexanoate and the P:O ratios of the same preparations with glutamate.

Thus, to different extents in different disrupted tissues, processes tending to generate free fatty acids outweigh those removing these compounds. This effect is greatest with plant preparations, intermediate with those from insects and least with those from higher animals.

Effect of different concentrations and types of fatty acids on isolated mitochondria. A wide variety of fatty acids was released during the incubation of carrot homogenates at 4°, and almost all inhibited

oxygen uptake and phosphorylation by isolated carrot mitochondria prepared with added albumin and oxidizing succinate and malate. The extent of inhibition depended markedly on the concentration of the fatty acid (especially in the range 0.1–1.0 mM); it would also depend on the period during which the mitochondria are in contact with the fatty acid. Thus, although the highest concentration of free fatty acids in the homogenates was 0.11 mM (after incubation at 4° for 60 min.), it is likely that incubation in less than this concentration of fatty acid for 1 hr. (i.e. with mitochondria prepared without added albumin) inhibits oxygen uptake and phosphorylation almost as effectively as does incubation in 1 mM-fatty acid for 15 min. (cf. Tables 4 and 7). The extent of inhibition also appeared to depend on an interplay between the water-solubility of the fatty acid at pH 7.1 and the chain length; thus a certain size was necessary to produce the marked inhibitory effects that were observed only with C₁₂–C₁₈ fatty acids (see Dervichian, 1954). Within this range a progressive decrease in inhibition occurred with increasing chain length. The importance of unsaturation of the chain was possibly in increasing the solubility of acids of comparable size; thus oleic acid, linoleic acid and linolenic acid were usually more potent inhibitors than stearic acid. Some of the variability of the results with stearic acid and oleic acid may have been due to variations between experiments in physical properties influencing the solubility of the fatty acid preparation used.

Previous workers using animal mitochondria have tended to emphasize the ability of unsaturated fatty acids (especially oleic acid) to induce uncoupling of oxidative phosphorylation and swelling in mitochondria (Lehninger & Remmert, 1959; Hulsmann, Elliott & Slater, 1960; Emmelot, 1962). The present results indicate that the ability to uncouple oxidative phosphorylation and inhibit oxygen uptake is found with a wide variety of fatty acids, in agreement with the data of Ahmed & Scholefield (1961). These considerations, together with the very marked inhibition by fatty acids of dehydrogenases that are structurally bound to the mitochondria, suggests that the action of the fatty acids is to disrupt the mitochondrial membrane by a detergent action (cf. Baker, Northcote & Peters, 1962). It is also possible that, with fatty acid concentrations that are not great enough to disrupt the mitochondrial-membrane structure completely, the fatty acids emulsify certain parts of the membrane, providing substrate readily available to mitochondrial lipases (Wojtczak & Lehninger, 1961); the generation of fatty acids under these conditions is likely to be autocatalytic.

Although the results do not establish that carrot mitochondria *in vivo* are subject to modification of

their oxidative properties by free fatty acids, it is conceivable that in some plant tissues the inhibitory effects of such acids are more important and indeed have a regulatory function. Thus decreased activity of the oxidases of the tricarboxylic acid cycle may be important in diverting acetate units for carbohydrate synthesis by the glyoxylate cycle (Beevers, 1961) in germinating fatty seedlings.

SUMMARY

1. The concentrations of free fatty acids in carrot homogenates, prepared with or without added albumin and kept at 4° for 25 min., were 0.013 and 0.015 mM respectively, rising after 60 min. to 0.041 and 0.11 mM respectively; the concentrations of free fatty acids in the mitochondrial suspensions prepared from these homogenates were 1.1 and 0.56 mM respectively.

2. The main components of these fractions were oleic acid, stearic acid, palmitic acid and an unidentified short-chain compound whose concentration rose considerably on standing; the mitochondrial fraction (prepared with added albumin) also contained relatively high concentrations of polyunsaturated C₁₈ fatty acids.

3. The total fatty acids of tissue lipid and mitochondrial lipid consisted primarily of linoleic acid and palmitic acid; large proportions of octadeca-6,9-dienoic acid and an unidentified fatty acid (*R_T* 2.81) were also present in mitochondrial lipid.

4. Carrot mitochondria, prepared with added albumin and incubated with succinate, malate, citrate, α -oxoglutarate and NADH₂, gave P:O ratios of 1.6, 1.9, 2.0, 2.0 and 1.3 respectively; comparable preparations isolated without albumin give P:O values of less than 0.1, and, with the same substrates, *q_o* values 15, 64, 43, 5, 9 and 47% respectively of the corresponding values for mitochondria prepared with added albumin.

5. Oleic acid (0.1, 1 and 10 mM) inhibited oxygen uptake with succinate to a greater extent than with malate.

6. Lauric acid, oleic acid, linolenic acid, myristic acid and linoleic acid inhibited oxygen uptake of carrot mitochondria, prepared with added albumin and incubated with succinate, by more than 70%; smaller inhibitions were observed with palmitic acid (57%), stearic acid (32%), arachidic acid (17%) and butyric acid (nil).

7. The fatty acids most effective in inhibiting oxygen uptake were also most effective in lowering the P:O ratios; the inhibition of phosphorylation and oxygen uptake increased markedly with an increase in chain length from C₈ to C₁₂.

8. Malate-dehydrogenase activity was not inhibited by 1 mM-stearate or 1 mM-oleate; succinate-dehydrogenase activity was inhibited 39% by

both fatty acids; dihydronicotinamide-adenine dinucleotide dehydrogenase was inhibited by 85 % and 92 % by 1 mM-stearate and 1 mM-oleate respectively.

9. The results are discussed in relation to the general problems of isolating 'intact' plant mitochondria and the action of fatty acids in inhibiting mitochondrial functions.

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Identification of the D-Glucosides of Stigmasterol, Sitosterol and Campesterol in Tobacco and Cigarette Smoke

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Several sterols and sterol esters have been reported in tobacco and in cigarette smoke. Reports on the presence of stigmasterol, β -sitosterol and γ -sitosterol in tobacco and cigarette smoke have been cited in a review by Johnstone & Plimmer

(1959). The presence of these sterols in cigarette smoke has been confirmed by Kosak & Swinehart (1960). Kosak, Swinehart, Taber & Van Duuren (1957) and Grossman & Stedman (1958), in reporting the occurrence of free stigmasterol in