Effect of Protein and Fatty Acids on the Oxidation of Succinate and α -Oxoglutarate by Pea-Root Mitochondria

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The oxidation of succinate and α -oxoglutarate by pea-root mitochondria was increased by cytochrome *c* or bovine-plasma albumin (Stickland, 1960). If the mitochondria were first shaken at 25° much higher rates of oxygen uptake were obtained. Similar effects of protein on oxidative phosphorylation have been shown in mitochondria from insects (Wojtczak & Wojtczak, 1960; Lewis & Fowler, 1960) and from liver (Hülsmann, Elliott & Slater, 1960). The activity of U factor, an uncoupling agent found by Lehninger & Remmert (1959) in rat-liver mitochondria, was abolished by plasma albumin. Since long-chain fatty acids could be extracted from protein that had been incubated with these mitochondria, it was suggested that the protein reversed an inhibition by fatty acids, and some fatty acids were shown to be inhibitory.

A similar effect appears to be operative in pea roots. In the present paper a further study of the effect of these proteins and of several fatty acids is reported.

EXPERIMENTAL

Peas were grown under sterile conditions and mitochondria extracted as reported by Stickland (1960). The terminal 2 cm. of pea roots was homogenized in 1 vol. of 0.6M-sucrose in 0.05M-phosphate buffer, pH 6.8, at 0°. Occasionally other lengths of root were used. The homogenate was centrifuged at 1000*g* for 10 min. and the supernatant centrifuged again at 15 000*g* for 15 min. The precipitate, which was composed mainly of mitochondria, was washed by resuspension in 0.5 vol. of the homogenizing medium, centrifuged again and finally resuspended in 0.5 vol. of medium.

In some experiments, after the second centrifuging the mitochondria were washed twice with 0.35 vol. of the homogenizing medium containing 3% (w/v) bovine-plasma albumin, and then washed once with 0.5 vol. of homogenizing medium and resuspended as above. All volumes are relative to the initial homogenizing volume.

Oxygen uptake was measured at room temperature (21–23°) by a polarographic method with a stationary platinum electrode sheathed with a thin polythene sheet (see Stickland, 1960). The reaction medium contained 0.05M-phosphate buffer, pH 6.8, 0.1 ml. of mitochondrial suspension and other additions as stated, in a total volume of 0.5 ml.

Nitrogen estimations were done by the method of Sunderland, Heyes & Brown (1957) on the 'acid-insoluble matter' obtained by the procedure of Werkheiser &

Bartley (1957). Oxygen uptake is expressed as q_{O_2} (μ l. of oxygen/mg. of N/hr.).

Preparation of ether extracts. Extracts of mitochondria, of bovine-plasma albumin that had been incubated with mitochondria and then precipitated with 2% (v/v) perchloric acid, and of the supernatant from this precipitation were prepared by shaking the material with four lots of 2 ml. of diethyl ether at 30°. The ether extracts were pooled and evaporated to dryness under a stream of nitrogen, and the residue was suspended or dissolved in water. Usually the weight of extracted material added to the reaction medium was equivalent to the weight originally present in 0.3 ml. of mitochondrial suspension.

Special chemicals. The following were used: bovine-plasma albumin and bovine γ -globulin (Armour Laboratories); diphosphopyridine nucleotide and linolenic acid (L. Light and Co. Ltd., Colnbrook, Bucks.); adenosine diphosphate and cytochrome *c* (Sigma Chemical Co., St Louis, Mo., U.S.A.); linoleic acid (British Drug Houses Ltd.).

RESULTS

Effect of cytochrome *c* on succinate oxidation. Addition of cytochrome *c* up to a concentration of 0.1 mM had no effect on the endogenous oxygen uptake of either unshaken mitochondria or mitochondria that were first shaken in a test tube in a water bath at 25° for 30 min. (Fig. 1*A*). There was very little oxidation of succinate in the absence of added cytochrome *c*, but as the cytochrome *c* concentration was increased the rate of oxidation rose, the increase with shaken mitochondria being much greater than that with unshaken mitochondria.

Effect of bovine-plasma albumin. Similar results were obtained with bovine-plasma albumin (Fig. 1*B*). The difference in q_{O_2} for succinate obtained with unshaken and shaken mitochondria was more marked than with cytochrome *c*. Bovine-plasma albumin had a similar effect on the oxidation of α -oxoglutarate (Table 1). There was only a very slow rate of oxidation of α -oxoglutarate by unshaken mitochondria but this was increased by bovine-plasma albumin. With shaken mitochondria there was appreciable oxidation of α -oxoglutarate and this was increased very considerably by bovine-plasma albumin, but in all cases the rate of oxygen uptake was less than with succinate as substrate.

Fig. 1. Effect of cytochrome *c* and bovine-plasma albumin on succinate oxidation by unshaken and shaken mitochondria. The reaction medium contained 0.05 M-phosphate buffer, pH 6.8, 0.1 ml. of mitochondrial suspension and succinate (10 mM) and cytochrome *c* (A) or bovine-plasma albumin (B) as indicated, in a total volume of 0.5 ml. ●, Endogenous, unshaken mitochondria; ○, endogenous, shaken mitochondria; ■ plus succinate, unshaken mitochondria; □, plus succinate, shaken mitochondria.

Table 1. Effect of bovine-plasma albumin on the oxidation of α -oxoglutarate by unshaken and shaken mitochondria

Reaction medium contained 0.05 M-phosphate buffer, pH 6.8, 0.1 ml. of mitochondrial suspension and additions as below in a total volume of 0.5 ml.

Additions	q_{O_2}	
	Unshaken mitochondria	Shaken mitochondria
None	490	510
α -Oxoglutarate (10 mM)	510	760
Bovine-plasma albumin (5 mg./ml.)	520	490
α -Oxoglutarate (10 mM) + bovine-plasma albumin (5 mg./ml.)	710	1070

Ether extract of mitochondria. An ether extract of pea-root mitochondria inhibited succinate oxidation. For this experiment the mitochondria were washed twice with homogenizing medium containing 3% (w/v) bovine-plasma albumin, and then once with homogenizing medium alone to remove excess of albumin. Mitochondria prepared in this way oxidized succinate at a rapid rate without the addition of bovine-plasma albumin, and the addition of 1.5 mg. of albumin/ml. did not affect the rate (Table 2). Ether extract corresponding to 0.3 ml. of mitochondrial suspension had no effect on the endogenous rate but resulted in 90% inhibition of succinate oxidation. This inhibition

Table 2. Effect of ether extract of mitochondria on the oxidation of succinate by unshaken mitochondria

Mitochondria were prepared in a sucrose-phosphate medium, washed twice with a medium containing 3% (w/v) bovine-plasma albumin and washed once with and finally resuspended in sucrose-phosphate. Reaction medium contained 0.05 M-phosphate buffer, pH 6.8, 0.1 ml. of mitochondrial suspension (unshaken) and additions as below in a total volume of 0.5 ml. The amount of mitochondrial extract originating from 0.3 ml. of mitochondrial suspension was used in each case.

Additions	q_{O_2}	
	No extract	Mitochondrial extract
None	530	550
Succinate (10 mM)	1160	610
Bovine-plasma albumin (1.5 mg./ml.)	530	560
Succinate (10 mM) + bovine-plasma albumin (1.5 mg./ml.)	1170	1160

was completely reversed by the addition of bovine-plasma albumin.

Ether extract of bovine-plasma albumin. A mitochondrial preparation was suspended in an equal volume of 3% (w/v) bovine-plasma albumin at room temperature for 5 min. The mitochondria were removed by centrifuging and the protein was precipitated from the supernatant with 2% (v/v) perchloric acid. Ether extracts were prepared (see Experimental section) of the sedimented mitochondria, of the precipitated albumin and of the final supernatant.

The effect of the extract of the albumin on the oxidation of succinate by mitochondria previously washed with bovine-plasma albumin is shown in Expt. 1 of Table 3. Approximately 50% inhibition of succinate oxidation was obtained and this inhibition was completely reversed by the addition of bovine-plasma albumin. The extract had no effect on the endogenous oxygen uptake.

Succinate oxidation was not inhibited by the ether extracts of the mitochondria that had been incubated with bovine-plasma albumin (see above), by the ether extract of the supernatant from the precipitated albumin or by a suspension in water of the precipitated albumin that had been extracted with ether.

If shaken albumin-washed mitochondria were used, the inhibition of succinate oxidation by the ether extract of bovine-plasma albumin was only about 20% (Table 3, Expt. 2). With α -oxoglutarate as substrate the inhibition by the albumin extract was about 75% with both unshaken and shaken mitochondria (Table 3, Expts. 3 and 4). All these

Table 3. *Effect of ether extract of bovine-plasma albumin on the oxidation of succinate and α -oxoglutarate*

Mitochondria were prepared and washed as noted in Table 2, and the reaction medium was as in Table 1. Concn. of succinate and α -oxoglutarate was 10 mM and that of bovine-plasma albumin was 1.5 mg./ml. The amount of bovine-plasma albumin extract originating from 0.3 ml. of mitochondrial suspension was used in each case.

Expt. no.	Mitochondria	Additions	q_{O_2}	
			No extract	Albumin extract
1	Unshaken	None	540	520
		Succinate	1430	980
		Bovine-plasma albumin	540	520
		Succinate + bovine-plasma albumin	1490	1460
2	Shaken	None	490	470
		Succinate	1270	1100
		Bovine-plasma albumin	470	460
		Succinate + bovine-plasma albumin	1280	1240
3	Unshaken	None	500	510
		α -Oxoglutarate	630	540
		Bovine-plasma albumin	500	500
		α -Oxoglutarate + bovine-plasma albumin	650	630
4	Shaken	None	490	470
		α -Oxoglutarate	670	520
		Bovine-plasma albumin	470	480
		α -Oxoglutarate + bovine-plasma albumin	690	680

Table 4. *Effect of bovine γ -globulin on the oxidation of succinate by unshaken and shaken mitochondria*

Reaction medium as given in Table 1. Concn. of succinate was 10 mM and of bovine γ -globulin 1.5 mg./ml.

Additions	q_{O_2}	
	Unshaken mitochondria	Shaken mitochondria
None	500	470
Succinate	600	680
γ -Globulin	510	490
Succinate + γ -globulin	690	780

inhibitions were completely reversed by bovine-plasma albumin.

An ether extract of 3% (w/v) bovine-plasma albumin alone inhibited succinate oxidation about 5%.

Effect of γ -globulin. Bovine γ -globulin, at a concentration of 1.5 mg./ml., also increased the rate of succinate oxidation by both unshaken and shaken mitochondria (Table 4), but the effect was very much less than that given by the same concentration of albumin. A higher concentration (5 mg./ml.) of γ -globulin caused inhibition of succinate oxidation.

Effect of fatty acids. The effect of five fatty acids on the oxidation of succinate by unshaken mitochondria is given in Table 5.

Stearate, octanoate, linoleate and linolenate all increased the oxygen uptake in the presence or absence of 10 mM-succinate. There was inhibition of succinate oxidation (*h-g* in Table 5) by 1 and 10 μ M-linoleate, but owing to the rise in the endo-

genous rate there was no inhibition of the total oxygen uptake in the presence of succinate. At a concentration of 10 μ M the other three fatty acids had little effect on succinate oxidation.

However, oleate depressed the total oxygen uptake in the presence of succinate, resulting in a marked inhibition of succinate oxidation (*f-e* in Table 5). Oleate (10 μ M) also increased the endogenous oxygen uptake.

Addition of bovine-plasma albumin tended to reverse the effects of stearate and oleate: wherever the oxygen uptake was increased by the fatty acids, albumin reduced it. However, in those cases where oleate decreased the oxygen uptake, addition of albumin raised it.

Large increases in the oxygen uptakes with or without succinate were caused by higher concentrations (up to 10 mM) of all five fatty acids. These were perhaps due to oxidation of the fatty acids themselves or to the uncoupling of oxidative phosphorylation, which fatty acids are now thought to do (see Lehninger & Remmert, 1959; Wojtczak & Wojtczak, 1960).

Different concentrations of ether extract. The effect of three concentrations of ether extract is also shown in Table 5. The two lower concentrations had no effect in the endogenous rate but inhibited succinate oxidation. The higher concentration, obtained from a different mitochondrial preparation, increased the oxygen uptake in both the absence and presence of succinate and caused some stimulation of succinate oxidation. Bovine-plasma albumin reversed the inhibition of succinate oxidation.

Table 5. *Effect of fatty acids on succinate oxidation*

Mitochondria were prepared and incubated as given in Table 2. Concn. of succinate was 10 mM and that of bovine-plasma albumin 1.5 mg./ml. Other additions as below. The oxygen uptake is expressed relative to that obtained in the presence of succinate alone, which was made equal to 100. The oxygen uptake due to the oxidation of succinate, after subtraction of the endogenous rate, is given in the third column in each case.

Concn. of fatty acid (μM)	Relative oxygen uptake with addition of									
	Stearate			Octanoate			Oleate			Concn. of ether extract (μg. dry wt./ml. of reaction medium)
	Alone a	With succinate b	With succinate + albumin b-a	Alone c	With succinate d	d-c	Alone e	With succinate f	With succinate + albumin f-e	
0	36	100	36	40	100	60	27	100	73	0
0.1	—	103	—	56	115	59	—	100	—	19
1	48	113	43	60	119	59	28	66	38	190
10	61	124	48	62	117	55	43	94	51	292*

Concn. of fatty acid (μM)	Relative oxygen uptake with addition of									
	Linoleate			Linolenate			Ether extract			Concn. of ether extract (μg. dry wt./ml. of reaction medium)
	Alone g	With succinate h	h-g	Alone i	With succinate j	j-i	Alone k	With succinate l	With succinate + albumin l-k	
0	53	100	47	41	100	59	36	100	37	0
0.1	53	100	47	41	100	59	39	73	40	19
1	66	103	37	51	109	58	36	82	36	190
10	80	109	29	77	140	63	55	128	73	292*

* From a different mitochondrial preparation from the lower concentrations.

Table 6. *Distribution of mitochondrial nitrogen and of ether-extractable material along the root*

Mitochondria were prepared from four consecutive 5 mm. sections of 475 pea roots. After removal of a sample for nitrogen determinations, each mitochondrial suspension was added to an equal volume of 3% (w/v) bovine-plasma albumin and an ether extract of the latter prepared as described in the Experimental section. The effect of each ether extract on the oxidation of succinate by mitochondria (prepared from root tips, length 2 cm.) washed with albumin was determined and is given in the Table under q_{O_2} . The amount of extract originating from 0.3 ml. of mitochondrial suspension was used in each case. The figures for % dry weight of root sections were obtained from a different lot of roots.

Section no.	Distance from root tip (mm.)	Fresh wt. of roots (g.)	Mitochondrial N		Dry wt. of ether extract (μ g.)	Dry wt. of extract (μ g./ μ g. of mitochondrial N)	q_{O_2}		Dry wt. of root sections (%)
			(μ g./475 sections)	(μ g./g. fresh wt. of roots)			Endogenous	With succinate	
1	0-5	2.05	375	183	<50	<0.2	450*	1120*	—
2	5-10	2.74	220	80	157	0.7	—	1130	16.1
3	10-15	3.19	248	78	379	1.5	470	1000	6.2
4	15-20	4.02	328	82	1375	4.2	550	760	6.5

* No ether extract added.

Table 7. *Effect of bovine-plasma albumin on the oxidation of succinate by mitochondria isolated from the tip (1 cm.) of pea roots*

Mitochondria were prepared from the tip (1 cm. only) of pea roots, and were not washed with albumin. The reaction medium was as in Table 1.

Additions	q_{O_2}	
	Unshaken mitochondria	Shaken mitochondria
None	550	510
Succinate (10 mM)	1160	1820
Bovine-plasma albumin (1.5 mg./ml.)	540	580
Succinate (10 mM) + bovine-plasma albumin (1.5 mg./ml.)	1170	1810

Mitochondrial nitrogen and ether-extractable material in pea-root sections. Mitochondria were prepared from consecutive 5 mm. sections of pea roots. An equal volume of 3% (w/v) bovine-plasma albumin was added to each mitochondrial suspension, and an ether extract of the albumin made as described in the Experimental section. Mitochondrial protein nitrogen, the dry weight of the ether extract and the inhibitory effect of the ether extract on succinate oxidation by mitochondria prepared from 2 cm. lengths of root and washed with albumin are reported in Table 6. There was a twofold increase in the fresh weight of the roots from sections 1 to 4. Both the mitochondrial nitrogen per gram fresh weight of roots and the percentage dry weight of the root sections decreased to under half their value from sections 1 to 2 and then remained approximately constant. The total dry weight of the ether extract increased very greatly from sections 1 to 4 and this was accompanied by an increasing inhibition of suc-

cinatc oxidation. The ether extract from section 4 also caused an increased oxygen uptake in the absence of succinate. The ratio of dry weight of extract to mitochondrial nitrogen increased at least 20 times from sections 1 to 4.

Since there was little ether-extractable material in the first two 5 mm. sections of the roots, it might have been expected that mitochondria prepared from 1 cm. sections should show no response to the addition of bovine-plasma albumin. This was in fact so, as indicated in Table 7. Addition of albumin had no effect on the oxygen uptake of either unshaken or shaken mitochondria.

Oxygen uptake on a dry-weight basis. Since many measurements of oxygen uptake are on a dry-weight basis, the dry weights of 2 cm.-long pea roots were determined to enable the results in this paper to be compared with others. With these dry-weight values, the endogenous oxygen uptake was about 0.5 μ l. of oxygen/mg. dry wt./hr., and with succinate values up to 3.5 μ l. of oxygen/mg. dry wt./hr. were obtained, depending upon the conditions.

DISCUSSION

The work reported in this paper suggests that a substance present in pea roots inhibits the oxidation of succinate and α -oxoglutarate by mitochondria isolated from these roots. This inhibition could be reversed by the addition of protein (cytochrome *c* or bovine-plasma albumin) and the inhibitor could be extracted from the protein that had been incubated with the mitochondria. Stimulation of the mitochondrial oxidations by protein thus appeared to be due to removal of inhibitor by the protein. The inhibitor, possibly in a combined form, may occur in other parts of the cell and only become associated with the mitochondria on disintegration of the root cells. That the amount of

inhibitor associated with the mitochondria varied so greatly from one region of the root to another (see Table 6) suggests that this might be so. Since most inhibitor was found in those areas of the root that have highly vacuolated cells, the inhibitor might be released on homogenization of the tissue either because it is in the vacuole or because the acids known to be in the vacuole bring about its formation by hydrolysis.

The work of Hülsmann *et al.* (1960) suggests that in animal mitochondria the uncoupler of oxidative phosphorylation is one (or more) unsaturated fatty acid. Of the fatty acids whose effects on pea-root mitochondria have been studied, only oleic acid resembles the inhibitor in its action of inhibiting the total oxygen uptake in the presence of succinate. A higher concentration of linoleate caused inhibition of succinate oxidation, but the total oxygen uptake was greater than that without linoleate. It is thus possible that the inhibitor is oleic or a closely related acid. According to Meara (1957), in most roots 80–90% of the fatty acids are unsaturated and consist mainly of oleic acid. The increased oxygen uptakes at high concentration of fatty acid are perhaps due partly to oxidation of the fatty acids themselves and partly to the uncoupling of oxidative phosphorylation, which fatty acids are now thought to do (see Lehninger & Remmert, 1959; Wojtczak & Wojtczak, 1960).

Particles isolated from cotton hypocotyls have been found to contain a substance that inhibits succinate oxidation (Throneberry, 1960), and this effect is partly reversed by plasma albumin. This inhibitory substance might be similar to the pea-root inhibitor.

The figures for distribution of mitochondrial nitrogen along the pea root are similar to those obtained for maize roots by Lund, Vatter & Hanson (1958). By using the values of Brown & Broadbent (1950) for the number of cells in pea roots, the amount of mitochondrial nitrogen per cell in sections 1 and 2 of the root is 3.6 and 8.2 $\mu\text{g.}$, which represent 2 and 3% of the protein nitrogen per cell respectively. However, these are only minimal values since it is almost certain that a quantitative extraction of the mitochondria was not obtained. The ratio of mitochondrial nitrogen (per gram fresh weight of roots) to percentage dry weight of the roots is approximately constant in all the sections.

SUMMARY

1. The oxidation of succinate and α -oxoglutarate by mitochondria from the terminal 2 cm. of pea roots was increased by cytochrome *c* or

bovine-plasma albumin. If the mitochondria were washed with albumin, the rates of oxidation were high and addition of albumin caused no further increase.

2. A diethyl ether extract of the mitochondria, or of bovine-plasma albumin that had been incubated with the mitochondria, inhibited the oxidation of both substrates by albumin-washed mitochondria, and this inhibition could be reversed by addition of albumin.

3. Shaking the mitochondria for $\frac{1}{2}$ hr. at 25° increased the rates of oxidation, but the effects of cytochrome *c*, bovine-plasma albumin and the ether extracts were still apparent.

4. Stearic, octanoic, oleic, linoleic and linolenic acids caused an increase of the oxygen uptake in the absence of succinate and in some cases a small inhibition or stimulation of succinate oxidation at high concentrations of fatty acid. The effect of ether extract was only similar to that produced by oleic acid, which brought about considerable inhibition of succinate oxidation at low concentration (μM).

5. Mitochondrial nitrogen (*a*) decreased and the amount of ether-extractable material (*b*) increased with increasing distance from the root tip. The ratio *b/a* increased 20-fold along the first 2 cm. of root. Oxidation of succinate by mitochondria isolated from the terminal 1 cm. of root was not affected by the addition of albumin.

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