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The nature of the stimulation of the respiratory chain of rat liver mitochondria by glucagon pretreatment of animals

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1. Studies on the cytochrome spectra of liver mitochondria from control and glucagon-treated rats in State 4, State 3 and in the presence of uncoupler are reported. 2. The stimulation of electron flow between cytochromes c_1 and c observed previously [Halestrap (1978) *Biochem. J.* **172**, 399–405] was shown to be an artefact of Ca^{2+}-induced swelling of mitochondria. 3. When precautions were taken to prevent such swelling, glucagon treatment was shown to enhance the reduction of cytochromes c, c_1 and b_{558} in both State 3 and uncoupled conditions with either succinate or glutamate + malate as substrate. An increase in the reduction of cytochromes b_{562} and b_{566} was also seen in some, but not all, experiments. 4. In State 4 with succinate but not glutamate + malate as substrate, cytochromes c, c_1, b_{558}, b_{562} and b_{566} showed increased reduction. 5. Glucagon stimulated oxidation of duroquinol and palmitoylcarnitine by intact mitochondria and of NADH by disrupted mitochondria. 6. No effect of glucagon on succinate dehydrogenase activity or the temperature-dependence of succinate oxidation could be detected. 7. Glucagon enhanced the inhibition of the respiratory chain by colletotrichin, but not antimycin or 8-heptyl-4-hydroxyquinoline *N*-oxide. 8. These results are interpreted in terms of a primary stimulation by glucagon of the 'Q cycle' [Mitchell (1976) *J. Theor. Biol.* **62**, 827–367] within Complex III (ubiquinol:cytochrome *c* oxidoreductase) and a secondary site of action involving stimulation of electron flow into Complex III from the ubiquinone pool. 9. Ageing of mitochondria, hyperosmotic treatment or addition of 20mM-benzyl alcohol opposed the effects of glucagon treatment on cytochrome spectra and colletotrichin inhibition of respiration. 10. These results support the hypothesis that glucagon exerts its effects on the mitochondria by perturbing the membrane structure.

Yamazaki (1975) was the first person to describe the enhanced rate of ADP- or uncoupler-stimulated respiration of liver mitochondria isolated from glucagon-treated rats. Only oxidation of substrates entering the respiratory chain before cytochromes *b* was stimulated, the rate of ascorbate oxidation in the presence of *NNN'N'*-tetramethyl-*p*-phenylenediamine remaining unchanged. These observations have been confirmed and extended in several laboratories (Titheradge & Coore, 1976*a*,*b*; Halestrap, 1978*a*,*b*; Siess & Wieland, 1978, 1979; Titheradge *et al.*, 1978; Titheradge & Haynes, 1979; Yamazaki *et al.*, 1980) and cannot be explained by changes in substrate permeability, since stimulation is observed in submitochondrial particles and when duroquinol is used as a lipid-soluble artificial substrate (Titheradge *et al.*, 1978; Titheradge & Haynes, 1979).

Previous work from this laboratory (Halestrap, 1978*b*) suggested that the locus of the hormonal stimulation of respiration was between cytochromes c_1 and c. However, more recent work (Halestrap, 1981) has demonstrated that liver mitochondria from glucagon-treated rats are less susceptible to damage by Ca^{2+}-induced swelling than those from control animals. Such swelling has been reported to cause impairment of respiration through loss of cytochrome *c* (Chappell & Crofts, 1965) and should also cause a cross-over in the respiratory chain between cytochromes c_1 and c. It seemed possible, therefore, that the previous observations on the effects of glucagon on mitochondrial cytochrome spectra (Halestrap, 1978*b*) might be artefacts caused

Abbreviation used: Complex III, ubiquinol:cytochrome *c* oxidoreductase.

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by differential damage by Ca^{2+} contaminating incubation media to which insufficient chelating agents were added. The experiments reported in the present paper confirm these suspicions and provide new spectral studies performed in the presence of Ca^{2+} chelators. Similar studies are reported on the effects of 20mM-benzyl alcohol, mitochondrial ageing and hyperosmotic treatment of mitochondria, all processes which effectively reverse the changes in mitochondrial function induced by glucagon (A. E. Armston & A. P. Halestrap, unpublished work). The results suggest that all these treatments act at the same site in the respiratory chain and are best interpreted in terms of stimulation of the 'Q cycle' (Mitchell, 1976). These conclusions were supported by the use of a novel inhibitor of the respiratory chain, colletotrichin, a phytotoxic metabolite of the fungus *Colletotrichum nicotianae* (Suzuki *et al.*, 1976). In contrast with results of other workers (Siess & Wieland, 1978, 1979; Titheradge & Haynes, 1979), no consistent effect of glucagon treatment on succinate dehydrogenase activity was observed.

Experimental

Materials

The sources of all chemicals and biochemicals was as given in Halestrap (1978*a*). Antimycin A, 2-heptyl-4-hydroxyquinoline *N*-oxide, duroquinone and snake venom (from the king cobra, *Ophiophagus hannah*) were from Sigma (London) Chemical Co. Ltd., Poole, Dorset BH17 7NH, U.K. and collectotrichin was generously given by Professor A. Suzuki, Department of Agricultural Chemistry, University of Tokyo, Bunkyo-ku, Tokyo, Japan. Reduction of duroquinone to duroquinol was performed as described by Titheradge & Haynes (1979). Hormonal treatment of rats and isolation of liver mitochondria were performed as described previously (Halestrap, 1978*a*). Particular care was taken to avoid contamination of mitochondria with haemoglobin. Where cytochrome spectra were to be measured under State-3 conditions, albumin (10mg/ml) was present in the initial homogenization medium to prevent fatty acid binding to the mitochondria and so cause limitation of ADP-stimulated respiration by inhibition of the adenine nucleotide translocase (see Vignais, 1976; La Noue & Schoolwerth, 1979). Mitochondria were disrupted by freezing and thawing three times in isolation medium, a procedure giving maximal rates of exogenous NADH oxidation with minimal loss of cytochrome *c*.

Methods

Measurement of mitochondrial respiratory-chain activity was performed polarographically at 30°C as described previously (Halestrap, 1978*a*). The medium contained 125mM-KCl, 10mM-Mops (4-morphilinepropanesulphonic acid), 7mM-Tris base, 2mM-potassium phosphate and 2mM-potassium EGTA at pH 7.2. Succinate dehydrogenase activity was assayed by the method of Arrigoni & Singer (1962) in buffer containing 10mM-potassium phosphate, 0.5mM-phenazine methosulphate, 20μg of 2,6-dichloroindophenol/ml, 1mM-KCN and 10mM-potassium succinate at pH 7.2. The mitochondrial membrane potential was measured with ^{86}Rb in the presence of valinomycin as described previously (Halestrap, 1978*a*), whereas the pH gradient was determined by the distribution of D-[^{14}C]lactate (Halestrap, 1978*a*). Both measurements involved accurate measurement of mitochondrial volume (Halestrap, 1978*a*) and for all measurements the buffer contained 250mM-sucrose, 2mM-sodium phosphate, 2mM-sodium EGTA, 10mM-Mops, 7mM-Tris base, 0.05mM-RbCl, 0.2mM-KCl, 2mM-Tris succinate, 0.5mM-lithium D-lactate and 1μg of valinomycin/ml.

Ca^{2+}-dependent swelling of mitochondria was carried out in a Pye–Unicam SP.8100 split-beam spectrophotometer. To 5ml of buffer containing 125mM-KCl, 2.5mM-potassium phosphate, 2.5mM-potassium succinate, 10mM-Mops and 7mM-Tris base, pH 7.4, were added mitochondria (approx. 10mg of protein). After vortex-mixing the mitochondrial suspension was divided between two cuvettes in the spectrophotometer and the A_{535} recorded. After obtaining a baseline, $CaCl_2$ (10nmol of Ca^{2+}/mg of mitochondrial protein) was added to one of the cuvettes and the ΔA_{535} continuously recorded.

Mitochondrial ageing with Ca^{2+} was performed at 37°C for 5min in sucrose medium (300mM-sucrose, 10mM-Tris / HCl, 2mM-EGTA, 6mM-calcium EGTA, pH 7.4) at a protein concentration of approx. 75mg/ml. Ageing was terminated by addition of a large excess of ice-cold isolation medium and the mitochondria re-sedimented by centrifugation. Control mitochondria were subject to the same treatment in the absence of calcium EGTA.

Measurement of cytochrome spectra

Cytochrome spectra were studied at room temperature (22°C) in the same medium as used for oxygen-electrode experiments, with a protein concentration of approx. 10mg/ml and a cuvette path-length of 1cm. Oxygenation was achieved by addition of 5mM-H_2O_2. A purpose-built split-beam spectrophotometer was used which scanned at up to 10nm/s, taking 24 data-points per nm. Data was processed by a Hewlett–Packard 9845s computer interfaced to the analog output of the spectrophotometer by means of a 12-bit digital-to-analog converter housed in a Hewlett–Packard 6940B

Multiprogrammer. The monochromator stepper motor was also controlled by the computer and each step initiated analog-to-digital conversion and the acquisition of one data-point. Elimination of noise was achieved by using suitable electronic filters on the analog output of the spectrophotometer and by a smoothing routine applied after data acquisition. This routine fits cubic polynomial equations to sequential and overlapping 6 nm portions of the scan by least-squares regression and then joining the smoothed curves together by the curve of sines, ignoring the first and last 0.5 nm sections of each individual fitted curve (see Pollard, 1977). Such a procedure is capable of discriminating between the sharpest cytochrome peaks observed and unwanted noise of lower frequency than could be removed by the electronic filters. The spectrophotometer was also used to measure spectra at −170°C with a cuvette path-length of 2 mm as described previously (Halestrap, 1978*b*). For greater precision under these conditions, scanning of spectra was repeated four times in quick succession and the results meaned. In all experiments shown the reference cuvette contained rotenone and no added substrate, whereas the sample cuvette contained added substrate and other additions as indicated. Within any one experiment, protein concentrations were closely matched and the amount of uncoupler added was kept at a constant ratio to the protein concentration (0.25 nmol/mg of protein). This concentration of uncoupler was sufficient to stimulate respiration fully but not so great as to cause excessive oxidation of the *b* and *c* cytochromes. In Figs. 2–5 below the normal sequence of events was to obtain a baseline with mitochondria present in both cuvettes and then to add rotenone to the reference cuvette and substrate to the sample cuvette to achieve State 4. After obtaining a spectrum, ADP was added to the sample cuvette and a State-3 spectrum scanned, followed by uncoupler addition to the sample cuvette and rescanning.

All spectra shown are typical of at least six experiments (effects of glucagon) or three experiments (treatments *in vitro*). All spectra shown were subjected to baseline correction.

Results and discussion

Effects of Ca^{2+}-dependent swelling on mitochondrial respiration

Exposure of liver mitochondria to low concentrations of Ca^{2+} in the presence of phosphate leads to massive swelling and inhibition of respiration (Chappell & Crofts, 1965). This process is accompanied by loss of cytochrome *c* and may be reversed by addition of EGTA and ATP (Crofts & Chappell, 1965). Recent work from this laboratory has demonstrated that liver mitochondria from glucagon-treated rats are more resistant to this damage caused by Ca^{2+} than those from control rats (Halestrap, 1981). In Fig. 1 and Table 1, data are presented suggesting that the previous report from this laboratory that glucagon stimulates the respiratory chain between cytochromes c_1 and *c* was an artefact of this differential sensitivity to Ca^{2+}. Fig. 1 shows the time course of Ca^{2+}-induced swelling and its reversal by ATP in the presence of EGTA, whereas Table 1 provides parallel data on maximal rates of uncoupled respiration measured at various times during swelling and contraction. Swelling inhibited the rate of oxidation of all substrates tested, whereas ATP-induced contraction restored some of this lost activity. Addition of exogenous cytochrome *c* restored the rate of oxidation of ascorbate in the presence of *NNN'N'*-tetramethyl-*p*-phenylenediamine to control values, implying that respiration is limited by loss of cytochrome *c*, as suggested by Chappell & Crofts (1965). This was confirmed spectrophotometrically, since Ca^{2+}-induced swelling was shown to induce a cross-over between cytochromes *c* and c_1 (results not shown). Oxidation of succinate and glutamate + malate could not be fully restored to control values by cytochrome *c* addition (results not shown), which reflects

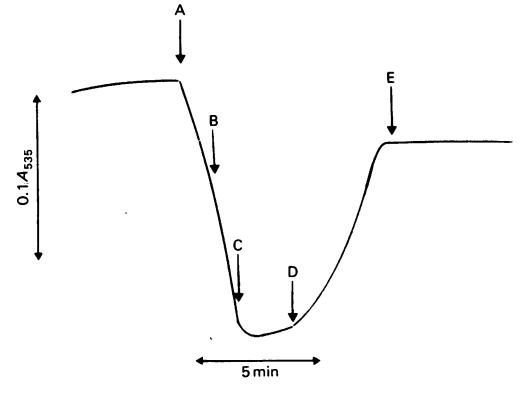

Fig. 1. *Time course of Ca^{2+}-dependent swelling of mitochondria and its reversal by ATP*

Mitochondria (approx. 2 mg of protein/ml) were incubated at 25°C in oxygen-electrode buffer (see the Experimental section) in the presence of 5 mM-succinate or glutamate + malate, but no added EGTA. Swelling was initiated by addition of 40 μM-calcium EGTA (at A) and monitored by the decrease in A_{535}. Addition of 1 mM-EGTA was made at C and 1 mM-ATP at D. In parallel experiments, 2 mM-EGTA was added at times A–E and the rate of oxygen uptake in the presence of uncoupler measured as recorded in Table 1. The trace shown is with succinate as substrate, but is typical of many experiments with glutamate + malate as substrate.

Table 1. *Effects of Ca^{2+}-induced mitochondrial swelling on respiratory-chain activity*

Rates of oxygen uptake were studied polarographically as described in the Experimental section. Where present, succinate was at 5mM, glutamate at 5mM, malate at 2mM, ascorbate at 10mM, *NNN'N'*-tetramethyl-*p*-phenylenediamine (TMPD) at 0.3mM, rotenone at 0.1μg/ml and cytochrome *c* at the various concentrations shown. Respiration was uncoupled by addition of 0.5μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. The conditions A–E represent the stages of swelling and contraction shown in Fig. 1. The experiment shown is typical of three similar experiments.

	Rate of oxygen uptake in the presence of substrates shown (ng-atoms of O_2/min per mg of protein)					
				Ascorbate + TMPD + cytochrome *c*		
Condition	Succinate + rotenone	Glutamate + malate	[Cytochrome *c*] (μg/ml) ...	0	20	50	100
A	123	75		144	156	156	160
B	—	37		73	110	148	176
C	21	5		39	90	132	176
D	—	12		52	90	115	150
E	72	55		104	119	153	163

an effect of ageing of mitochondria on oxidation of these substrates (A. E. Armston & A. P. Halestrap, unpublished work).

Effects of glucagon treatment on mitochondrial cytochrome spectra

In view of the possibility that previous studies on the nature of the glucagon effect on the respiratory chain may have been distorted by the effects of Ca^{2+}-dependent swelling, new spectrophotometric studies were made. In Figs. 2–5 the cytochrome spectra of liver mitochondria from control and glucagon-treated rats are shown. Spectra were recorded under State 4, State 3 and uncoupled conditions with both succinate (Figs. 2, 4 and 5) and glutamate + malate (Figs. 3 and 5) as substrate. Particular care was taken to ensure that the protein concentration was the same for control and experimental mitochondria and to maintain the ratio of uncoupler to mitochondrial protein constant at 0.25nmol of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone/mg of mitochondrial protein (see the Experimental section). Spectra were scanned at room temperature, except for those reported in Fig. 4, where samples were frozen at −170°C before scanning spectra as outlined in the Experimental section. Although some extra resolution of cytochromes *c* and *b* did occur at this temperature, little additional information could be derived from these spectra which was not apparent at room temperature. It is also possible that freezing respiring mitochondria in liquid N_2 may not be sufficiently rapid to prevent some perturbation of the spectra, although comparison of spectra at the two temperatures suggested no major changes. In the discussion following, the cytochromes to be considered with the wavelength of their room temperature and −170°C absorption maxima given in parentheses are cytochromes *c* (550, 546.5nm), c_1 (554, 550.5nm), *b* (558, 555.5nm), *b* (562, 559.5nm) and *b* (566, 563.5nm). Consideration of the functional and spatial relationships of these cytochromes are given in the following paper (Halestrap, 1982).

The data presented in Figs. 2–5 clearly indicate that, in the uncoupled state and in State 3 (ADP present) with both succinate and glutamate + malate as substrates, the effect of glucagon was to increase the reduction of both *b* and *c* cytochromes [Fig. 2(ix), Fig. 3(ix), Fig. 4(iii), Fig. 5*a*(i) and Fig. 5*b*(i)]. The spectra at −170°C (Fig. 4) indicate that glucagon may cause some extra reduction in all the *b* cytochromes as do the room-temperature spectra of Fig. 3(ix). However, in other experiments [Figs. 2(vii) and 2(ix), and Figs. 5(*a*)(i) and 5(*b*)(i)], only cytochrome b_{558} appeared to show increased reduction. In experiments on six different batches of control and glucagon-treated mitochondria the increased reduction of cytochromes *c*, c_1 and b_{558} was always observed whether succinate or glutamate + malate were used as substrates. Increased reduction of cytochromes b_{562} and b_{566} (combined peak at 564nm) was observed on only three occasions, however. The reason for this variability is not certain, but may reflect the existence of a second site of glucagon action on the respiratory chain whose response to the hormone is variable. Evidence for this is outlined below.

Evidence for a second site of glucagon action

Under State-4 conditions in the presence of succinate (but not glutamate + malate), increased reduction of cytochromes *c*, c_1, b_{558}, b_{562} and b_{566} was observed in mitochondria from glucagon-treated animals [Fig. 2(viii)]. However, under these conditions the flow of electrons through this region of the respiratory chain would be expected to be

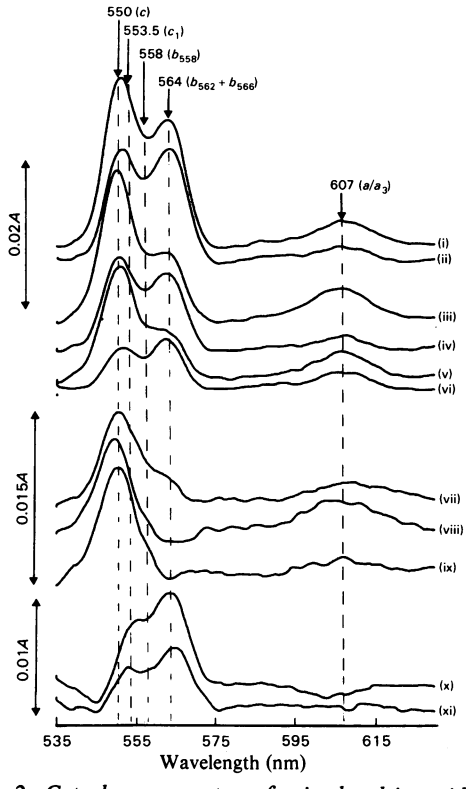

Fig. 2. *Cytochrome spectra of mitochondria oxidizing succinate*

Cytochrome spectra were measured in State 4 (i, ii), State 3 (iii, iv) and with uncoupler present (v, vi) as described in the Experimental section. Mitochondria were from control (ii, iv, vi) and glucagon-treated (i, iii, v) animals and were incubated at 10 mg of protein/ml. 5 mM-Succinate and 10 μg of rotenone/ml were present in the sample cuvette. Scans (vii)–(ix) represent difference spectra of glucagon-control mitochondria in State 4 (vii), State 3 (viii) and with uncoupler present (ix). Difference spectra of State 4 − State 3 are also shown for control (xi) and glucagon-treated (x) mitochondria. Abbreviations used: *c*, c_1 . . ., cytochromes *c*, c_1 . . . etc.

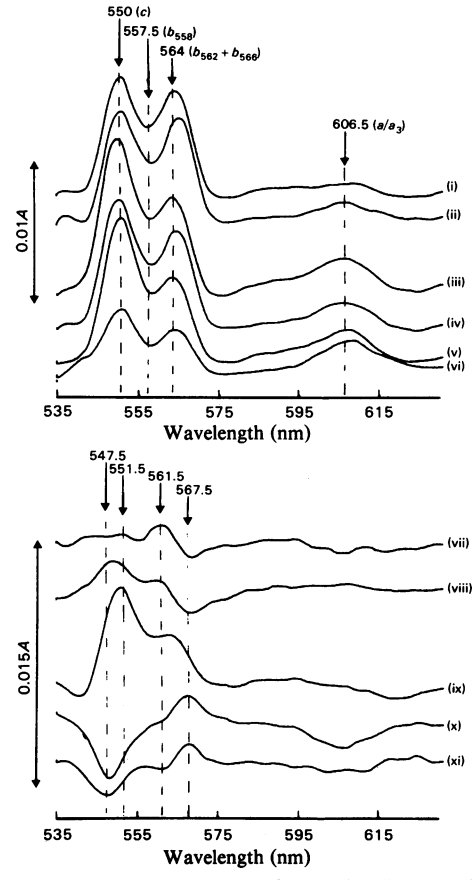

Fig. 3. *Cytochrome spectra of mitochondria oxidizing glutamate + malate*

The Experimental procedure and conditions were identical with those of Fig. 2, except that 5 mM-glutamate + 2 mM-malate replaced succinate and rotenone in the sample cuvette. For abbreviations, see Fig. 2.

restricted by the protonmotive force rather than by the step which limits electron flow under uncoupled conditions. To explain this effect of glucagon on the State-4 spectra seems to require an additional site of glucagon action, and two observations in the literature are of interest in this context. Firstly, it has been reported that liver mitochondria from glucagon-treated rats can maintain a higher protonmotive force in State 4 than those from control rats (Titheradge & Coore, 1976*b*; Prpic *et al.*, 1978). Such an increase in protonmotive force would be expected to cause an increase in the reduction state of the *b* and *c* cytochromes [see, for example, Fig. 8 of the following paper (Halestrap, 1982)]. However, we have been unable to detect such a change in protonmotive force under State-4 conditions (Table 2 and Halestrap, 1978*a*). The reasons for this discrepancy are uncertain, but could reflect the failure by these previous workers to measure accurately either the intramitochondrial volume, which increases with glucagon treatment (Halestrap *et al.*, 1980; Halestrap, 1981), or the pH gradient. Determination of this latter parameter is frequently distorted by metabolism of the pH marker molecules used (Halestrap, 1978*a*). Thus it appears that the increase in reduction state of the *b* and *c*

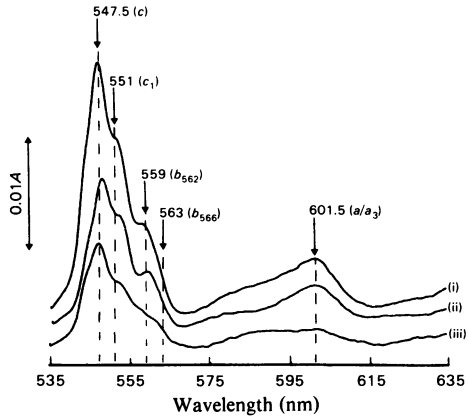

Fig. 4. *Spectra of uncoupled mitochondria oxidizing succinate measured at −170°C*

Conditions for measuring spectra were identical with those of Figs. 2(v) and (vi), except that the protein concentration was 20 mg/ml and the samples were frozen in liquid N_2 before scanning spectra as described in the Experimental section. Mitochondria were from control (ii) and glucagon-treated (i) rats and were uncoupled as described in the Experimental section. The difference spectrum (glucagon − control) is shown in (iii). For abbreviations see Fig. 2.

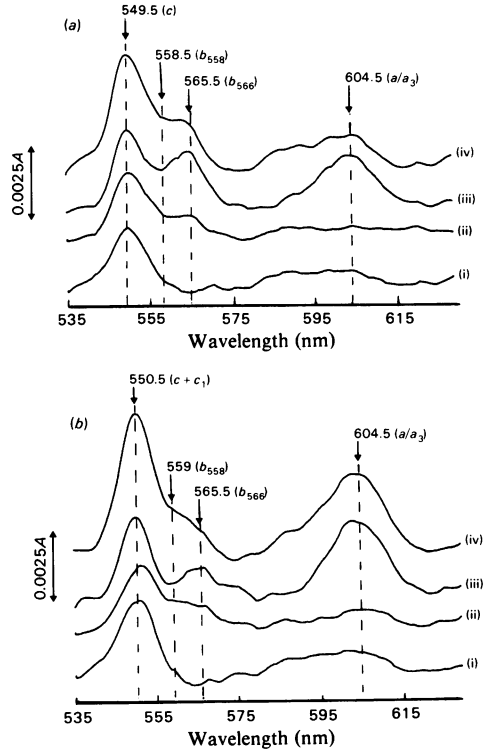

Fig. 5. *Difference spectra of uncoupled mitochondria oxidizing succinate or glutamate + malate under various conditions*

The uncoupled cytochrome spectra of mitochondria in the presence of glutamate + malate (*a*) or succinate (*b*) were measured as described in Figs. 2 and 3. Mitochondria from control and glucagon-treated animals were used. In some experiments the latter mitochondria were also subject to ageing, hyperosmotic treatment (addition of 250 mM-sucrose to medium) or addition of 20 mM-benzyl alcohol. The difference spectra represent glucagon − control (i), glucagon − aged glucagon (ii), glucagon − hyperosmotic glucagon (iv) and glucagon − glucagon + 20 mM-benzyl alcohol (iii).

cytochromes in State 4 cannot be explained by changes in the protonmotive force.

The second observation in the literature which is of interest is the stimulation of succinate dehydrogenase that has been reported (Siess & Wieland, 1978, 1979; Titheradge & Haynes, 1979). This would stimulate electron flow into Complex III and so induce the increased reduction state of the *b* and *c* cytochromes observed. It is also quite consistent with the proposal that glucagon influences the disposition of the mitochondrial inner membrane (Halestrap, 1981), since succinate dehydrogenase is an integral membrane protein. However, I have been totally unable to observe such changes in succinate dehydrogenase activity, despite assaying the enzyme at different temperatures and different concentrations of phenazine methosulphate (see Table 2). It is not easy to account for this discrepancy. It is possible that the assays of Siess & Wieland (1978, 1979) and Titheradge & Haynes (1979) allowed electron flow through Complex III to the monitoring dye rather than directly from succinate dehydrogenase. In the assays reported here, neither antimycin nor rotenone affected the enzyme rate, indicating that no such electron transfer was occurring.

There is further evidence against succinate dehydrogenase being activated by glucagon to a significant extent. Malonate titration of uncoupled mitochondria oxidizing succinate (5 mM) showed that no significant inhibition of oxidation occurred until about 15 μM-malonate was present. At 30 μM-malonate, inhibition was greater in mitochondria from glucagon-treated animals and the glucagon effect was diminished (Table 2). This implies that, in intact mitochondria, succinate dehydrogenase is unlikely to be the rate-limiting step in succinate oxidation that is stimulated by glucagon. Moreover, as indicated by others (Siess & Wieland, 1978;

Table 2. *Effects of glucagon treatment on various mitochondrial parameters*

Measurements of the various parameters were made as described in the Experimental section. In all cases the glucagon-induced stimulation of uncoupled succinate oxidation is given as an indication of the extent of glucagon action. NADH oxidation by disrupted mitochondria was corrected for antimycin-insensitive oxidation. Statistical significance of the difference between control and experimental conditions estimated by Students *t* test are: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Parameter	Units	Parameter value: Control	Parameter value: Glucagon-treated	Effect of glucagon on uncoupled succinate oxidation (% stimulation)	No. of observations
1. Succinate dehydrogenase activity	nmol of succinate/min per mg of protein				
At 37°C		236.1 ± 21.6	274 ± 29.2	50 ± 11.2**	10
At 30°C		164.1 ± 8.8	172.8 ± 11.3	42 ± 4.8***	29
At 16°C		60.0 ± 4.5	46.6 ± 2.2	33 ± 9.2*	5
At 30°C but no phenazine methosulphate		31.4 ± 1.9	32.8 ± 2.6	30 ± 5.8**	5
2. Rate of oxygen uptake by uncoupled mitochondria with:	ng-atoms of O_2/min per mg of protein				
Palmitoyl-L-carnitine (20 μM) + L-malate (2 mM)		45.0 ± 4.0	77.6 ± 7.9**	32 ± 7.8**	6
Duroquinol (0.5 mM)		162 ± 5.8	217 ± 4.4***	33 ± 4.9*	3
NADH (1 mM) (disrupted mitochondria)		66.1 ± 5.4	88.5 ± 6.2**	35 ± 5.5***	19
3. Inhibition of uncoupled succinate oxidation by 30 μM-malonate	(%)	10.9 ± 2.8	20.7 ± 1.9***	44 ± 5.4***	6
4. Components of the protonmotive force	(mV)				
$pH_{in} - pH_{out}$		19.7 ± 2.03	16.4 ± 1.43		
Membrane potential		−159.4 ± 1.26	−158.9 ± 0.97	34 ± 4.1**	4
Protonmotive force		−179.1 ± 2.32	−175.3 ± 2.18		

Titheradge & Haynes, 1979), any change in succinate dehydrogenase cannot account for effects of glucagon on the oxidation of other substrates. In agreement with Titheradge & Haynes (1979), I have observed stimulation of duroquinol oxidation induced by glucagon and also palmitoylcarnitine oxidation (Table 2). Table 2 also contains data indicating that antimycin-sensitive NADH oxidation by frozen and thawed mitochondria is faster in mitochondria from glucagon-treated animals, implying that stimulation of the oxidation of substrates providing NADH occurs beyond NADH production. These results confirm the conclusions of Titheradge *et al.* (1978), who used submitochondrial particles to demonstrate that stimulated substrate transport into mitochondria could not account for the effects of glucagon.

Taken together the foregoing observations suggest that glucagon stimulation of respiration is located within Complex III itself, but that an additional stimulation of electron flow from succinate may also be involved. The site of this stimulation is discussed below, under 'Conclusions'.

Effects of glucagon on the inhibition of the respiratory chain by colletotrichin

Further information about the site of action of glucagon within Complex III comes from studies with the inhibitor of the respiratory chain, colletotrichin. This is a phytotoxic metabolite of the fungus *Colletotrichum nicotianae* (Suzuki *et al.*, 1976), whose structure has some resemblance to ubiquinone. Aceytl-collectotrichin, an acetylated derivative also occurring naturally, has been shown to inhibit the respiratory chain somewhere within Complex III at a site distinct from the antimycin site (Foucher *et al.*, 1974). In the following paper (Halestrap, 1982) I provide evidence to suggest that colletotrichin behaves similarly and inhibits respiration by perturbing the interaction between a

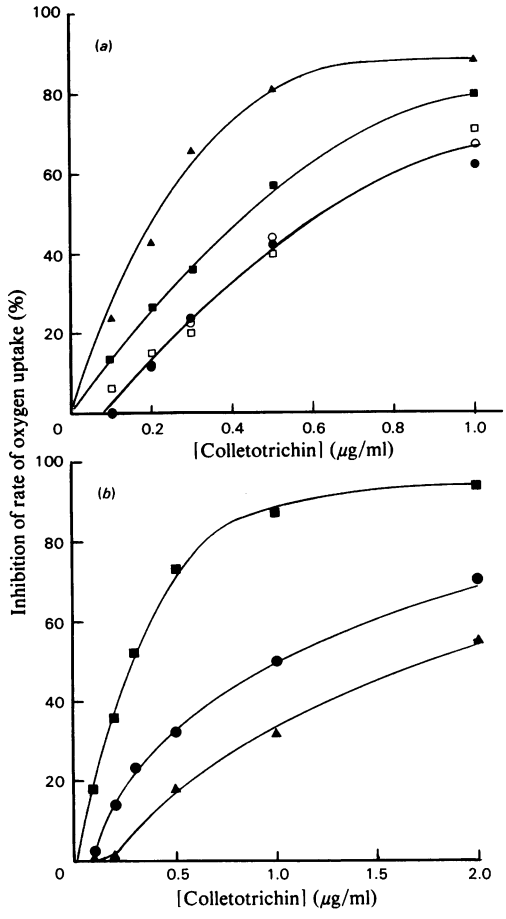

Fig. 6. *Colletotrichin inhibition of respiration by intact uncoupled mitochondria and disrupted mitochondria*
In (*a*), oxidation of succinate by intact uncoupled mitochondria was studied as described in the Experimental section and in the legend to Table 2. Increasing concentrations of colletotrichin were added sequentially from a 1mg/ml solution in ethanol. Mitochondria were from control rats (●, ▲) or glucagon-treated rats (■, □, ○) which in one case (○) were aged before use as described in the Experimental section. For □, 20mM-benzyl alcohol was present, whereas for ▲ the osmolarity of the buffer was decreased to 100mosM by decreasing the concentration of KCl. Rates of O_2 uptake in the absence of colletotrichin (in ng-atoms of O_2/min per mg of protein) were 70 (●), 115 (■), 162 (▲), 80 (○), 68 (□). In (*b*), mitochondria were disrupted by freezing and thawing and the rate of antimycin-sensitive NADH-dependent O_2 uptake was measured as described in the Experimental section and in the legend to Table 2. Disrupted mitochondria from control (●) and glucagon-treated (■) animals were used, and in one case (▲) the latter were exposed to 20μg/ml of snake venom for 5min before assay. Rates of O_2 uptake in the absence of colletotrichin were 126 (■), 83 (●) and 67 (▲) ng-atoms of O_2/min per mg of protein.

ubisemiquinone radical and cytochrome b_{558}. The data of Figs. 6(*a*) and 6(*b*) demonstrate that this inhibition was more potent in mitochondria from glucagon-treated rats, whether intact (Fig. 6*a*) or disrupted (Fig. 6*b*). Furthermore, treatment of mitochondria with 20mM-benzyl alcohol or ageing with Ca^{2+} decreased the sensitivity of respiration to inhibition by colletotrichin to control values. Hypo-osmotic treatment of control mitochondria, on the other hand, made them more sensitive to inhibitor. Such treatments also mimic or reverse glucagon action on other mitochondrial function in a parallel manner (A. E. Armston & A. P. Halestrap, unpublished work). Direct exposure of disrupted mitochondria to 20μg of snake venom/ml (rich in phospholipase A_2) has a similar effect to ageing mitochondria with Ca^{2+} and impaired both antimycin-sensitive NADH oxidation and colletotrichin inhibition of respiration (Fig. 6*b*).

The data presented on the effects of glucagon and treatments *in vitro* on collectotrichin inhibition of the respiratory chain suggest a site of action of glucagon on the lipid domain in close proximity to the site of action of colletotrichin within Complex III. No effects of glucagon or treatments *in vitro* on the sensitivity of respiration to antimycin or 2-heptyl-4-hydroxyquinoline *N*-oxide, both inhibitors of electron flow through Complex III, were detected (results not shown). A scheme accounting for these observations is proposed in the following paper (Halestrap, 1982). In outline it is suggested that colletotrichin binds to cytochrome b_{558} and that the action of glucagon is to influence the lipid environment around this cytochrome. This stimulates both electron flow from cytochrome b_{562} to cytochrome b_{558} and the binding of colletotrichin.

Effects of mitochondrial ageing, intramitochondrial volume and benzyl alcohol on cytochrome spectra

Data are shown in Fig. 5 on the effects of ageing of mitochondria, incubation in hyperosmotic medium or exposure to 20mM-benzyl alcohol on the cytochrome spectra of uncoupled mitochondria oxidizing succinate. In each case the treatment caused an oxidation of cytochromes c_1 and c relative to the untreated mitochondria, similar to that observed when control mitochondria are compared with those from glucagon-treated animals. This was true whether succinate or glutamate + malate was the respiratory substrate. However, in the case of cytochromes b_{562} and b_{566} the treatments with hyperosmotic media, benzyl alcohol and, to a lesser extent, mitochondrial ageing, caused an oxidation not apparent in mitochondria from control mitochondria within the same experiment. As outlined above, the changes in reduction of the *b*-cytochromes with glucagon treatment were variable, and in some experiments significant reduction of b_{562} and

b_{566} was apparent after glucagon treatment [Fig. 3(ix) and Fig. 4]. Thus the effects of the various treatments *in vitro* are quite compatible with their sharing with glucagon a common locus of action within Complex III.

Temperature-dependence of the glucagon effect on the respiratory chain

Many mitochondrial enzymes associated with the mitochondrial inner membrane have activities whose temperature-dependence show characteristic breaks in the Arrhenius plots at the transition temperatures of the membrane phospholipids (see, for example, Raison & McMurchie, 1974; Halestrap, 1975; Parenti-Castelli *et al.*, 1979; Sondergaard, 1979; Abuirmeileh & Elson, 1980). A major change in phospholipid structure would be expected to change both the transition temperature and break point in the Arrhenius plot (McMurchie & Raison, 1979; Abuirmeileh & Elson, 1980). For oxidation of succinate by rat liver mitochondria, large changes in the break point of the Arrhenius plot can be observed by altering the composition of dietary fat and consequently the membrane phospholipids (McMurchie & Raison, 1979; Innis & Clandinin, 1981). In Fig. 7 the effects of temperature on the oxidation of succinate and ascorbate + *NNN'N'*-tetramethyl-*p*-phenylenediamine by liver mitochondria from control and glucagon-treated rats is shown. It is clear that stimulation of succinate oxidation occurred at all temperatures studied and that there is no change in the transition temperature. This implies that any change in membrane structure induced by glucagon must be small and insufficient to cause gross effects on membrane behaviour. Such a conclusion is supported by the lack of any effect of glucagon on either the midpoint potential of any of the mitochondrial cytochromes or their pH-dependence (A. P. Halestrap, unpublished work). Major changes in the mitochondrial phospholipid composition might be expected to alter these parameters (Yu *et al.*, 1979).

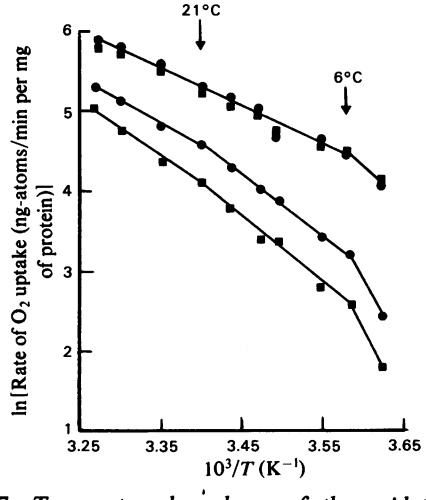

Fig. 7. *Temperature-dependence of the oxidation of succinate and ascorbate + NNN'N'-tetramethyl-p-phenylenediamine by uncoupled mitochondria*

Oxidation of 5 mM-succinate (lower two traces) or 10 mM-ascorbate + 0.3 mM-*NNN'N'*-tetramethyl-*p*-phenylenediamine by uncoupled mitochondria from control (■) or glucagon-treated (●) mitochondria was measured polarographically as described in the Experimental section and the legends to Tables 1 and 2. The experiment shown is typical of four such experiments.

Conclusions

The present results suggest that the previous observation (Halestrap, 1978*b*) that glucagon stimulates the respiratory chain between cytochromes c_1 and c was an artefact caused by Ca^{2+}-dependent swelling. This process causes loss of cytochrome c from mitochondria (Fig. 1, Table 1 and Chappell & Crofts, 1965) and is inhibited in mitochondria from glucagon-treated animals (Halestrap, 1981). Rather it appears that the main site of glucagon action on the respiratory chain lies within Complex III itself. Thus the cytochrome spectra of mitochondria from glucagon-treated animals oxidizing either succinate or glutamate + malate, whether uncoupled or in State 3, show cytochromes c, c_1 and b_{558} to be more reduced than in control mitochondria (Figs. 2–6). The reduction states of cytochromes b_{562} and b_{566} are sometimes, but not always, increased by glucagon treatment. These data are consistent with a site of stimulation of electron flow within the 'Q cycle' (Mitchell, 1976), and a more detailed account of the pathways of electron flow involved are given in the following paper (Halestrap, 1982). It is suggested that glucagon may influence the lipid environment of Complex III such that electron flow from cytochromes b_{562} to b_{558} via ubiquinol is enhanced. Two additional experimental approaches have been used which support this hypothesis.

Firstly, inhibition of respiration by colletotrichin, a ubiquinone analogue (Foucher *et al.*, 1974; Suzuki *et al.*, 1976), is enhanced in mitochondria (intact or disrupted) from glucagon-treated animals (Fig. 6). In the following paper (Halestrap, 1982) data are presented which indicate that this inhibitor acts by displacing ubisemiquinone from cytochrome b_{558}. Secondly, perturbation of the mitochondrial membrane by changing the intramitochondrial volume, ageing of mitochondria or addition of 20 mM-benzyl alcohol all cause reversion of glucagon-treated mitochondria to those resembling control mitochondria with respect to both their cytochrome

spectra (Fig. 5) and their inhibition by colletotrichin (Fig. 6).

Enhancement of the state of reduction of cytochromes *b* and *c* is also seen in mitochondria from glucagon-treated animals under State-4 conditions with succinate, but not with glutamate + malate, as substrate (Figs. 2 and 3). Under these conditions there is no enhancement of respiration induced by glucagon (Yamazaki, 1975; Titheradge & Coore, 1976*b*; Halestrap, 1978*a*; Hughes & Barritt, 1978; Yamazaki *et al.*, 1980), nor a change in mitochondrial protonmotive force (Table 2). This suggests that the enhancement of the reduction of cytochromes *c* and *b* is due to an additional effect of glucagon influencing the flow of electrons from succinate into Complex III. Reports from two laboratories suggest that succinate dehydrogenase may be activated by glucagon (Siess & Wieland, 1978, 1979; Titheradge & Haynes, 1979), but the present results (Table 2) do not support this conclusion. Furthermore, the glucagon-induced enhancement of duroquinol oxidation (Titheradge & Haynes, 1979, and Table 2) and palmitoylcarnitine oxidation (Table 2) by intact mitochondria and NADH oxidation by submitochondrial particles (Titheradge *et al.*, 1978) and disrupted mitochondria (Table 2) suggest that the major site of action of glucagon is within Complex III itself and not at the level of succinate dehydrogenase. Nevertheless, a mechanism of action of glucagon on the mitochondria that involves perturbation of the membrane lipids (Halestrap, 1981) would be quite capable of influencing electron flow at more than one point in the respiratory chain.

Perhaps the most likely additional site of modulation of electron flow is at the level of the ubiquinone pool linking Complex III with the various substrate dehydrogenases. Such a lipid-soluble intermediate as ubiquinone could be markedly influenced by the lipid environment (Ragan, 1978). Electrons from succinate enter this pool without any form of respiratory control, whereas for glutamate + malate an additional coupling site is available. This accounts for the greater state of reduction of cytochromes *b* and *c* in State 4 with succinate as substrate than with glutamate + malate [Figs. 2(i) and (ii); cf. Figs. 3(i) and (ii)], despite the redox potential of the $NADH/NAD^+$ couple being lower than that of succinate/fumarate. A consequence of this is that State-4 rates of succinate oxidation are considerably faster than those of glutamate + malate oxidation (see, for example, Yamazaki, 1975; Halestrap, 1978*a*; Titheradge & Haynes, 1979). If glucagon stimulates flow through the ubiquinone pool into Complex III, increased reduction of cytochromes *b* and *c* will only be observed if flow of electrons into the ubiquinone pool is not rate-limiting. For succinate this will be true, but for glutamate + malate, with its additional coupling site, it may well not be so. This would explain why glucagon treatment causes changes in the State-4 spectra when succinate but not glutamate + malate are substrates.

The changes in membrane structure required to induce changes in respiratory-chain activity must be very limited, since we have been unable to detect changes in the major phospholipid components after glucagon treatment (A. E. Armston & A. P. Halestrap, unpublished work). They must also be insufficient to cause any major changes in the activation energy or transition temperatures for succinate oxidation (Fig. 7). An understanding of the lipid–protein interactions within Complex III and how they may influence the kinetics of electron flow must await future research. It is known, however, that phospholipids play a vital role in the functioning of this region of the respiratory chain (Yu *et al.*, 1978).

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