The mechanism of the hormonal activation of respiration in isolated hepatocytes and its importance in the regulation of gluconeogenesis

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1. The effects of hormones on the cytochrome spectra of isolated hepatocytes were recorded under conditions of active gluconeogenesis from L-lactate. Glucagon, phenylephrine, vasopressin and valinomycin, at concentrations that caused stimulation of gluconeogenesis, increased the reduction of the components of the cytochrome bc_1 complex, just as has been observed in liver mitochondria isolated from glucagon-treated rats [Halestrap (1982) *Biochem. J.* **204**, 37–47]. The effects of glucagon and phenylephrine were additive.
2. The time courses of the increased reduction of cytochrome c/c_1 and NAD(P)H/NAD(P)⁺ caused by hormones, valinomycin, A23187 and ethanol were measured by dual-beam spectrophotometry and fluorescence respectively.
3. Ethanol (14 mM) produced a substantial rise in NAD(P)H fluorescence, β -hydroxybutyrate/acetoacetate and lactate/pyruvate ratios, no change in cytochrome c/c_1 reduction, a 10% decrease in O₂ consumption and a 60% decrease in gluconeogenesis.
4. Glucagon, phenylephrine and vasopressin caused a substantial and transient rise in NAD(P)H fluorescence, but a sustained increase in cytochrome c/c_1 reduction and the rates of O₂ consumption and gluconeogenesis.
5. The transience of the fluorescence response was greater in the absence of Ca²⁺, when the cytochrome c/c_1 response also became transient.
6. The fluorescence response was smaller and less transient, but the cytochrome c/c_1 response was greater, in the presence of fatty acids. Both responses were greatly decreased by the presence of 1 mM-pent-4-enoate.
7. Valinomycin (2.5 nM) caused a decrease in NAD(P)H fluorescence coincident with an increase in cytochrome c/c_1 reduction and the rate of gluconeogenesis and O₂ consumption.
8. A23187 (7.5 mM) caused increases in both NAD(P)H fluorescence and cytochrome c/c_1 reduction.
9. The effects of hormones and valinomycin on the time courses of NAD(P)H fluorescence, cytochrome c/c_1 reduction and light-scattering by hepatocytes were compared with those of 0.5 μ M-Ca²⁺ or 1 nM-valinomycin on the same parameters of isolated liver mitochondria.
10. It is concluded that hormones increase respiration by hepatocytes in a biphasic manner. An initial Ca²⁺-dependent activation of mitochondrial dehydrogenases rapidly increases the mitochondrial [NADH], which is followed by a volume-mediated stimulation of fatty acid oxidation and electron flow between NADH and cytochrome c .
10. Amytal (0.5 mM) was able to reverse the effects of hormones on the reduction of cytochromes c/c_1 and the rates of gluconeogenesis and O₂ consumption without significantly lowering tissue [ATP].
11. It is concluded that coincident activation of fatty acid oxidation and the respiratory chain is a primary site of hormonal stimulation of gluconeogenesis from C₃ precursors in the starved hepatocyte.

INTRODUCTION

It is well established that glucagon, vasopressin and α -agonists stimulate O₂ uptake by isolated hepatocytes and the perfused liver (Exton *et al.*, 1972; Dehaye *et al.*, 1981; Reinhart *et al.*, 1982, 1984; Buxton *et al.*, 1982; Binet & Claret, 1983; Blackmore *et al.*, 1983; Sies *et al.*, 1983; Taylor *et al.*, 1983). This stimulation of respiration cannot be accounted for by a decrease in the protonmotive force, since this is not observed either by direct experimentation (Strzelecki *et al.*, 1984) or by measurement of the mitochondrial ATP/ADP ratio, which has been shown to reflect the protonmotive force and so to regulate the respiratory chain (Nicholls & Bernson, 1977; Hansford, 1980; Wanders *et al.*, 1981; Williamson *et al.*, 1981). Indeed there are several reports that the mitochondrial ATP/ADP ratio may increase after hormone treatment (Bryla *et al.*, 1977; Siess *et al.*, 1977, 1978; Titheradge *et al.*, 1979; Titheradge & Haynes, 1980a,b; Aprille *et al.*, 1982). Another potential

mechanism for increasing the rate of the respiratory chain would be to increase the supply of NADH and thus the NADH/NAD⁺ ratio (see Hansford, 1980; Erecinska & Wilson, 1982). There are several reports to show that those hormones that stimulate respiration also increase the NADH/NAD⁺ ratio as determined by using both fluorescence and the β -hydroxybutyrate/acetoacetate ratio, but the effect appears to be transient, unlike that on respiration (Parrilla *et al.*, 1975; Sugano *et al.*, 1980; Balaban & Blum, 1982; Blackmore *et al.*, 1983; Kimura *et al.*, 1984). These observations might suggest that some other factor is involved in the stimulation of the respiratory chain. This would seem quite likely, in view of the observed increase in respiratory-chain activity of liver mitochondria isolated from glucagon- or adrenaline-treated rats (see Halestrap *et al.*, 1983; Halestrap, 1986). Furthermore, we have previously demonstrated that such an activation of the respiratory chain can be induced by an increase in matrix volume of the same order as that observed in hepatocytes treated with hormones

(Halestrap, 1982; Armston *et al.*, 1982; Quinlan *et al.*, 1983). In the present paper we seek to demonstrate that such an activation of the respiratory chain does occur and that it may account for the sustained increase in O_2 consumption in the face of a transient rise in NADH/NAD⁺ ratio and little or no change in mitochondrial ATP/ADP ratio. We also provide evidence that this activation of the respiratory chain is an essential component of the hormonal stimulation of gluconeogenesis in the starved rat hepatocyte.

EXPERIMENTAL

Materials

The sources of all chemicals and biochemicals were as given in the preceding paper (Halestrap *et al.*, 1986), where details are also given of the preparation and incubation of isolated rat liver cells and mitochondria. For routine experiments bovine serum albumin (fraction V) was obtained from Park Scientific Ltd., Northampton, U.K., whereas, for experiments at controlled fatty acid concentrations, essentially fatty-acid-free fraction V albumin was obtained from Sigma Chemical Co., Poole, Dorset, U.K. Oleate was added to the albumin to give the required concentration, by the method of Ross *et al.* (1967b). Pent-4-enoic acid was from Lancaster Synthesis Ltd., Morecambe, Lancs., U.K. Amytal (sodium amylbarbitone) was obtained from Eli Lilly, Basingstoke, Hants., U.K.

Methods

Unless otherwise stated, all hepatocyte incubations contained 10 mM-L-lactate, 1 mM-pyruvate and 20 mg of dialysed bovine serum albumin/ml.

Measurement of hormone-induced changes in the cytochrome spectra of hepatocytes. Hepatocytes (20 mg of protein/ml) were incubated for 20 min at 37 °C in an orbital shaking water bath with constant gassing (O_2/CO_2 , 19:1), and then 3.5 ml samples were transferred to both reference and sample cuvettes of a computerized split-beam spectrophotometer (Halestrap, 1982). Both cuvettes were vigorously stirred with an overhead stirrer in an atmosphere of O_2/CO_2 (19:1) and were maintained at 37 °C. It was demonstrated that glucose production occurred at rates similar to those under more conventional conditions and was hormone-sensitive. After 5 min further incubation in the cuvettes, the required hormone was added to the sample cuvette, and incubation continued for 5 min. At this point the stirrer was stopped and the cytochrome spectra were scanned four times between 530 and 630 nm. The scan rate was 10 nm/s, with 12 data points per nm being recorded. The total scan time was 40 s, which was rapid enough to prevent hypoxia or significant sedimentation of cells. Data were stored on a Hewlett-Packard 9845s computer, which allowed averaging and smoothing of the data as detailed previously (Halestrap, 1982).

Measurement of the time courses of the hormone-induced reduction of cytochrome c/c_1 and NADH. Cytochrome c/c_1 reduction was measured in a double-beam spectrophotometer which was a modified form of the split-beam spectrophotometer used above. The wavelength pair was 551.5–540 nm. The reduction state of NADH was measured both by double-beam spectrophotometry

(356–375 nm) or by 90° fluorimetry. For the latter, the same basic instrument was used, but the light source and signal processing were modified as required. Excitation and emission wavelengths were set by using suitable Wratten filters at 350–380 nm and 420–480 nm respectively. Hepatocytes were incubated at 12 (spectrophotometric studies) or 4 (fluorimetric studies) mg of protein/ml for 20 min as described above before a 3.5 ml sample was transferred to the cuvette of a double-beam spectrophotometer or fluorimeter. Incubation was continued with constant stirring, and 10 data points/s were recorded and averaged to give a final stored trace with 1 data point/s. Additions were made to the cuvette as required through a suitable injection port. Estimation of the steady-state reduction of cytochromes c/c_1 was made by fully oxidizing and reducing the cytochromes by addition of 2 µg of antimycin/ml and 0.5 mM-cyanide respectively.

Measurement of metabolites and rates of O_2 uptake and gluconeogenesis. Rates of glucose production were measured by the glucose oxidase assay as described previously (Quinlan *et al.*, 1983). Rates of O_2 consumption were measured in a purpose-built sealed thermostatically regulated plastic container with a Clark-type O_2 electrode. The electrode was calibrated by using standardized NADH and mitochondria made permeable to NADH by freezing and thawing. Hepatocytes were preincubated for 20 min at 20 mg of protein/ml as described above before transfer of 0.5 ml of the cell suspension to 2.0 ml of freshly oxygenated medium maintained at 37 °C in the O_2 -electrode vessel. After a steady rate of O_2 consumption was achieved (about 1 min), hormones were added as required. The ratios of β -hydroxybutyrate/acetate and lactate/pyruvate and the ATP concentration in the deproteinized extract were determined as described previously (Thomas & Halestrap, 1981).

RESULTS

Effects of hormones on the cytochrome spectra of isolated hepatocytes

The data of Fig. 1 show the effects of 0.1 µM-glucagon, 20 µM-phenylephrine and 2.5 nM-valinomycin on the cytochrome spectra of hepatocytes from 24 h-starved rats incubated under conditions of active gluconeogenesis from L-lactate. For comparison, data are also shown in Fig. 1 for the effects of glucagon treatment of rats on the spectra of subsequently isolated mitochondria oxidizing succinate in State 4 and State 3 (Halestrap, 1982). In the hepatocytes, the effect of hormone treatment is to increase the reduction state of the cytochromes b and c in a similar way to that in experiments with isolated mitochondria. Less detailed studies of the effects of glucagon on the cytochrome spectra of perfused rat liver have been performed by Kimura *et al.* (1984) and are in basic agreement with the results reported here.

Time course of the effects of hormones on the reduction of cytochrome c and the NAD(P)H fluorescence

Figs. 2 and 3 show the time courses of the increase in the reduction of cytochromes c/c_1 and of the NAD(P)H fluorescence caused by an hormone treatment of hepatocytes under various conditions. Statistical analysis of the effects observed is given in Table 1, which also

Fig. 1. Effects of hormone treatment of hepatocytes on their cytochrome spectra

Continuous lines represent the difference spectra between control and hormone-treated hepatocytes measured as described in the Experimental section. Each spectrum is the mean of scans from four experiments on different cell preparations. Additions made to the sample cuvette were $0.1 \mu\text{M}$ -glucagon (G), $20 \mu\text{M}$ -phenylephrine (P), both hormones together (G+P) or 2.5 nM -valinomycin (Val). The broken lines represent the difference spectra between liver mitochondria from glucagon-treated and control rats oxidizing succinate in State 4 (St4) or State 3 (St3) measured as described in Halestrap (1982).

includes measurements of the rates of O_2 consumption and gluconeogenesis under the same conditions. Glucagon ($0.1 \mu\text{M}$), phenylephrine ($20 \mu\text{M}$) and vasopressin (25 nM) all produced a rapid reduction of both NAD(P)H and cytochrome c/c_1 , which began within 1 s of hormone addition and was maximal at 20–30 s. Thereafter the fluorescence signal decreased again, especially after glucagon treatment, where the value 5 min after hormone addition was barely above basal, as observed by others (Williamson *et al.*, 1969b; Sugano *et al.*, 1980; Balaban & Blum, 1982; Kimura *et al.*, 1984). No such secondary decrease in the reduction state of cytochrome c/c_1 was observed, except when Ca^{2+} was omitted from the incubation medium. Under these conditions both the changes in NAD(P)H fluorescence and cytochrome c/c_1 reduction state were extremely transient [Figs. 2 and 3 (traces vii and viii), and Table 2]. The peak fluorescence changes induced by hormones were greater and more transient in the absence of fatty acid. Conversely the increases in cytochrome c/c_1 reduction were greater in the presence of fatty acids, even though the reduction state of cytochrome c/c_1 of the control cells was already increased from 12 to 18% by the presence of fatty acids (see also Sies & Brauser, 1980). Addition of phenylephrine after glucagon had completed its transient effects on fluorescence led to another increase in fluorescence, which was maintained at 5 min to a greater extent than that induced by phenylephrine alone. Similar additive effects of the two hormones were also seen on cytochrome c/c_1 reduction (Fig. 2), confirming the additive effects seen on the cytochrome spectra (Fig. 1).

Fig. 2. Time course of the effects of hormones on the increase in cytochrome c/c_1 reduction in hepatocytes incubated under various conditions

The experimental technique is outlined in the Experimental section. Hepatocytes (12 mg of protein/ml) were preincubated for 20 min at 37°C before placing them in the spectrophotometer. The incubation medium contained 10 mM-L-lactate, 1 mM-pyruvate and 20 mg of defatted albumin/ml and was supplemented with 1 mM-oleate or 1 mM-pent-4-enoate as indicated. Ca^{2+} was present at 2.5 mM, except in traces (v) and (vi), where 0.2 mM-EGTA was added just before the start of the recording. Glucagon ($0.1 \mu\text{M}$; GLUC) and phenylephrine ($20 \mu\text{M}$; PHEN) were added at the times shown. Quantitative analysis of the response to hormones may be found in Tables 1 and 2.

Fig. 3. Time course of the effects of hormones on the increase in NAD(P)H fluorescence in hepatocytes incubated under various conditions

Conditions were identical with those of Fig. 2, except that fluorescence was measured at a cell concentration of 6 mg/ml as described in the Experimental section. The β -hydroxybutyrate/acetoacetate ratios measured in the presence of 1 mM-oleate 10 min after hormone addition and given as means $\pm$ S.E.M. for four observations on parallel incubations were 0.70 ± 0.01 (control), 0.98 ± 0.02 (phenylephrine), 0.86 ± 0.03 (glucagon) and 1.34 ± 0.04 (glucagon + phenylephrine).

In Fig. 4 data are presented on the hormone-sensitivity of the fluorescence response. Although detailed analysis of these data was not performed, it appears that half-maximal and maximal fluorescence responses were achieved at about 0.2 μ M- and 1.0 μ M-phenylephrine and 1 nM- and 100 nM-glucagon respectively. The sensitivity of the cytochrome c/c_1 response to hormones could not be measured with accuracy because of the signal-to-noise ratio of the response.

Effect of ethanol, A23187 and valinomycin on NAD(P)H fluorescence and cytochrome c/c_1 reduction

The transience of the hormone changes in cytochrome c/c_1 reduction apparent when Ca^{2+} was removed from the medium (Table 2) implies a role for Ca^{2+} in this response. Fig. 5 shows that addition of 7.5 μ M-A23187 to hepatocytes to increase cytoplasmic Ca^{2+} to the same extent as with hormones causes a rise in both NAD(P)H fluorescence and cytochrome c/c_1 reduction. It should be noted that the intrinsic fluorescence of A23187 was corrected for, but required the sensitivity of the fluorimeter to be decreased in these experiments. We have shown in the preceding paper that this concentration of A23187 also causes mitochondrial swelling and activation of gluconeogenesis similar to that induced by hormones (Halestrap *et al.*, 1986). Another treatment of liver cells which is known to increase the NADH/NAD $^{+}$ ratio is addition of ethanol (14 mM), as shown in Fig. 5. There was a rapid and large rise in the fluorescence signal, but little or no rise in the reduction state of cytochrome c/c_1 . Nor was there a rise in O_2 consumption; rather there was a substantial fall in the rate of gluconeogenesis and about a 10% decrease in the rate of O_2 consumption (Fig. 5, and

Berry *et al.*, 1983a,b). Parallel measurements of the cytoplasmic and mitochondrial redox state by using the lactate/pyruvate and β -hydroxybutyrate/acetoacetate ratios respectively showed that these were increased greatly by ethanol treatment (see the legend to Fig. 5). Measurement of these metabolite ratios was also used to confirm the redox changes seen with hormone treatment (see the legend to Fig. 3). Changes in the redox state of cytoplasmic and mitochondrial compartments estimated in this way were similar to those obtained in many other laboratories (see, e.g., Williamson *et al.*, 1969b; Parrilla *et al.*, 1975; Siess *et al.*, 1977; Bucher & Sies, 1980; Berry *et al.*, 1983b).

It is of interest that the transient change in NAD(P)H fluorescence seen in the present studies and by others (Sugano *et al.*, 1980; Kimura *et al.*, 1984; Balaban & Blum, 1982) was not reflected in a transient change in the β -hydroxybutyrate/acetoacetate ratio (P. T. Quinlan & A. P. Halestrap, unpublished work; Williamson *et al.*, 1969b; Berry *et al.*, 1983a,b; Soboll *et al.*, 1984; Scholz *et al.*, 1984). This could indicate that the fluorescence changes observed are not reflecting the mitochondrial NADH/NAD $^{+}$ ratio as has been predicted (Williamson *et al.*, 1969b; Sugano *et al.*, 1980; Balaban & Blum, 1982; Kimura *et al.*, 1984). However, the use of recirculating media in perfusion studies and standard liver-cell incubation procedures (as opposed to perfusion) may well be responsible for this. The extracellular β -hydroxybutyrate/acetoacetate ratio is unlikely to re-equilibrate with the mitochondrial NADH/NAD $^{+}$ ratio with sufficient rapidity to allow the detection of transient changes in this parameter. In non-recirculating perfused livers, transient hormone-induced changes in β -hydroxy-

Table 1. Influence of fatty acid oxidation on the effects of hormones on gluconeogenesis, O₂ consumption, NAD(P)H fluorescence and cytochrome *c* reduction

Hepatocytes were incubated as described in the Experimental section in the presence or absence of 1 mM-oleate and 1 mM-pent-4-enoate as indicated; 10 mM-L-lactate, 1 mM-L-pyruvate and 20 mg of defatted albumin/ml were present in all incubations. Rates of gluconeogenesis, O₂ consumption, cytochrome *c* reduction state and NAD(P)H fluorescence were all measured after a 20 min preincubation period in the presence of all additions except hormones. The changes in cytochrome *c* reduction state given were measured 3 min after hormone addition, when a new steady state was obtained. However, the value at 30 s after hormone addition was not statistically different from that at 5 min. Values are presented as means $\pm$ S.E.M. for the numbers of observations (on different cell preparations) shown in parentheses. Note that for all hormone treatments the change in parameter value rather than the absolute value is given. For fluorescence measurements arbitrary units are used, the sensitivity of the fluorimeter and the cell concentrations being identical for all experiments. Statistical significance of hormone treatments versus the relevant controls was calculated by paired Student's *t* test (***) $P < 0.001$; ** $P < 0.01$; * $P < 0.05$, as was the significance of the effect of pent-4-enoate (†† $P < 0.01$; † $P < 0.05$). The effects of fatty acids were analysed by an unpaired *t* test (††† $P < 0.001$; †† $P < 0.01$; † $P < 0.05$). Abbreviations: n.a., not applicable; n.d., not determined.

Additions to incubation	Control value or change in parameter value caused by hormones				
	Rate of gluconeogenesis (nmol/min per mg of protein)	Rate of oxygen consumption (ng-atoms/min per mg of protein)	Cytochrome <i>c</i> reduction at 3 min (% change)	NAD(P)H fluorescence at 30 s (arbitrary units)	NAD(P)H fluorescence at 5 min (arbitrary units)
(a) No oleate					
Control value	3.67 $\pm$ 0.23 (11)	25.1 $\pm$ 2.0 (3)	11.7 $\pm$ 0.65 (2)	n.a.	n.a.
0.1 μ M-Glucagon	1.70 $\pm$ 0.21 (11)	6.0 $\pm$ 1.3 (3)*	1.79 $\pm$ 0.11 (5)	49.3 $\pm$ 6.1 (10)***	3.3 $\pm$ 1.3 (10)*
20 μ M-Phenylephrine	1.15 $\pm$ 0.14 (11)***	8.7 $\pm$ 0.8 (3)**	2.89 $\pm$ 0.24 (7)***	84.1 $\pm$ 4.2 (10)***	35.2 $\pm$ 6.3 (10)***
Glucagon + phenylephrine	1.095 $\pm$ 0.09 (4)***	n.d.	4.43 $\pm$ 0.34 (5)***	79.0 $\pm$ 8.2 (10)***	52.2 $\pm$ 5.8 (10)***
25 nM-Vasopressin	1.26 $\pm$ 0.30 (5)*	n.d.	2.8 $\pm$ 0.28 (2)	91.0 $\pm$ 10.9 (4)*	38.00 $\pm$ 9.7 (4)*
2.5 nM-Valinomycin	1.09 $\pm$ 0.20 (4)*	9.2 $\pm$ 0.5 (3)**	1.68 $\pm$ 0.36 (6)*	n.a.	-30.0 $\pm$ 2.8 (5)***
(b) 1 mM-Oleate					
Control value	6.77 $\pm$ 0.50 (14)†††	50.9 $\pm$ 3.0 (10)†††	18.0 $\pm$ 0.75 (2)†††	n.a.	n.a.
Glucagon	1.28 $\pm$ 0.20 (14)***	6.9 $\pm$ 0.41 (10)***	2.77 $\pm$ 0.34 (15)***†	19.9 $\pm$ 2.3 (12)***†††	7.0 $\pm$ 1.5 (12)***
Phenylephrine	1.39 $\pm$ 0.24 (14)***	8.5 $\pm$ 0.72 (10)***	4.43 $\pm$ 0.29 (14)***††	39.2 $\pm$ 3.6 (13)***†††	21.7 $\pm$ 1.7 (13)***
Glucagon + phenylephrine	1.41 $\pm$ 0.19 (11)***	10.6 $\pm$ 1.18 (5)***	6.61 $\pm$ 0.37 (14)***††	49.8 $\pm$ 5.2 (11)***††	37.8 $\pm$ 5.3 (11)***
Vasopressin	0.99 $\pm$ 0.40 (8)*	7.1 $\pm$ 0.58 (10)***	4.71 $\pm$ 0.48 (7)***	38.6 $\pm$ 6.0 (4)***††	20.3 $\pm$ 2.4 (4)***
Valinomycin	0.37 $\pm$ 0.19 (6)†	4.1 $\pm$ 0.49 (8)***†††	2.13 $\pm$ 0.27 (9)***	n.a.	-38.0 $\pm$ 5.0 (6)***
(c) 1 mM-Pent-4-enoate					
Control value	3.29 $\pm$ 0.25 (5)	29.1 $\pm$ 1.8 (3)†	n.d.	n.a.	n.a.
Glucagon	0.40 $\pm$ 0.05 (5)***††	8.6 $\pm$ 1.9 (3)*	0.77 $\pm$ 0.21 (2)†	14.0 $\pm$ 8.0 (2)††	1.5 $\pm$ 1.5 (2)
Phenylephrine	0.13 $\pm$ 0.09 (5)††	6.4 $\pm$ 2.3 (3)	1.33 $\pm$ 0.07 (2)*†	15.5 $\pm$ 4.5 (2)††	6.0 $\pm$ 1.0 (2)††
(d) 1 mM-Oleate + 1 mM-pent-4-enoate					
Control value	3.77 $\pm$ 0.29 (6)***†††	43.3 $\pm$ 2.9 (3)†	n.d.	n.a.	n.a.
Glucagon	0.44 $\pm$ 0.13 (6)***††	4.2 $\pm$ 0.74 (3)*	1.46 $\pm$ 0.28 (3)***††	8.5 $\pm$ 0.5 (3)***††	6.5 $\pm$ 2.5 (3)
Phenylephrine	0.47 $\pm$ 0.19 (6)††	4.1 $\pm$ 0.30 (3)**	1.68 $\pm$ 0.56 (3)††	11.00 $\pm$ 2.6 (3)††	8.7 $\pm$ 1.9 (3)†

Table 2. Hormonal changes in cytochrome *c* reduction and NAD(P)H fluorescence in the absence of extracellular Ca^{2+}

Hepatocytes were preincubated for 20 min in Ca^{2+} -free media containing 20 mg of defatted albumin/ml, 10 mM-L-lactate and 1 mM-pyruvate before transfer to a cuvette for absorption or fluorescence measurements. At 2 min before hormone addition 0.2 mM-EGTA was added to the incubation medium. Values are given as means $\pm$ S.E.M. for *n* cell preparations. Further details are given in the Experimental section. The statistical significance of the difference of hormone-treated from control cells was calculated by a paired Student's *t* test (***P* < 0.001; **P* < 0.01).

Hormone treatment	Change in cytochrome <i>c</i> reduction (%)			Fluorescence change		
	30 s	5 min	<i>n</i>	30 s	5 min	<i>n</i>
0.1 μM -Glucagon	$1.99 \pm 0.06^{**}$	0.06 ± 0.14	5	$36.3 \pm 1.9^{**}$	1.7 ± 4.4	3
20 μM -Phenylephrine	$2.02 \pm 0.33^*$	0.22 ± 0.10	5	$55.3 \pm 2.4^{**}$	1.3 ± 4.7	3
Glucagon + phenylephrine	$1.62 \pm 0.09^{**}$	0.06 ± 0.06	5	$59.7 \pm 2.6^{**}$	-3.3 ± 8.8	3
5 nM-Vasopressin	$2.17 \pm 0.24^*$	-0.14 ± 0.14	4	$66.0 \pm 6.0^*$	9.0 ± 1.0	2

Fig. 4. Effects of submaximal hormone concentrations on NAD(P)H fluorescence

Experimental conditions were the same as for Fig. 3, but glucagon (G) or phenylephrine (P) were added at the concentrations indicated. Defatted albumin was used in these experiments.

butyrate/acetoacetate ratios have been observed for phenylephrine (Haussinger & Siess, 1984), vasopressin and glucagon (H. Siess, unpublished work). Siess & Wieland (1980) have shown a transient effect of glucagon on this parameter in isolated hepatocytes. It is generally thought that fluorescence detects largely bound mitochondrial NAD(P)H, because of the fluorescence enhancement of such bound nucleotides, whereas absorbance detects the total NAD(P)H without discrimination (Avi-Dor *et al.*, 1962; Eastbrook, 1962; Bucher & Siess, 1980). This explains the relatively modest increase in fluorescence caused by ethanol addition in Fig. 5, despite the very large increase in lactate/pyruvate ratio and hence in cytoplasmic NADH/ NAD^+ . We have demonstrated changes in NAD(P)H absorbance ($A_{356-375}$) very similar to those obtained by fluorescence, as would be expected, but the fluorescence technique gave greater sensitivity, was more specific for the mitochondrial NAD(P)H pool and required the use of less cells. If it is accepted that the fluorescence changes observed are reflecting the mitochondrial NADH/ NAD^+ ratio, then it is clear that the

reduction state of the cytochromes *c* and c_1 does not necessarily reflect the mitochondrial NADH/ NAD^+ . This is even more apparent when 2.5 nM-valinomycin is added to hepatocytes, as shown in Fig. 5. Here a decrease in NAD(P)H fluorescence is accompanied by an increase in cytochrome *c*/ c_1 reduction. Both O_2 consumption and gluconeogenesis are simulated under these conditions (Table 1).

Effects of Ca^{2+} on the light-scattering, cytochrome *c*/ c_1 reduction and NAD(P)H fluorescence of isolated liver mitochondria

The data of Fig. 6 demonstrate that the time courses for the hormonal or valinomycin-induced stimulation of mitochondrial swelling, NAD(P)H fluorescence and cytochrome *c*/ c_1 reduction in the hepatocyte can be mimicked by addition of Ca^{2+} (about 1 μM final concn.) or 1 nM-valinomycin to isolated liver mitochondria. The isolated mitochondria were incubated in the presence of glutamate and malate to energize them, and 5 mg of defatted albumin/ml, 1 mM-MgATP and 0.5 mM-EGTA to maintain their stability during the incubation. They were kept in a state intermediate between State 3 and State 4 by the addition of glucose and hexokinase. It took 6–8 min for all three parameters to reach a steady state. During this initial phase the mitochondria were swelling (see Halestrap *et al.*, 1986), and both cytochrome *c* and NADH were becoming oxidized (results not shown). This may partially reflect loss of Ca^{2+} into the incubation medium containing EGTA and 5 mM- Na^+ (McCormack, 1985b), leading to inhibition of the dehydrogenases of the tricarboxylic acid cycle. The presence of 0.5 μM - Ca^{2+} from the start of the incubation diminished this oxidation. The data presented are for mitochondria preincubated for 8 min. Addition of Ca^{2+} back to the incubation to give a free $[\text{Ca}^{2+}]$ of about 1 μM produced a transient rise in NAD(P)H fluorescence, a stable increase in cytochrome *c*/ c_1 reduction and a decrease in light-scattering. When valinomycin was added, an oxidation of NAD(P)H was observed which was coincident with an increase in cytochrome *c*/ c_1 reduction and mitochondrial swelling. These studies *in vitro* show a very close parallel to the hormonal effects seen on isolated hepatocytes incubated in the absence of fatty acids. If succinate was added to the isolated mitochondrial incubations in order to mimic the production of FADH_2 rather than NADH in the initial reaction of fatty acid oxidation, the mitochondria behaved more like hepato-

Fig. 5. Effects of various agents on the time courses of NAD(P)H fluorescence and cytochrome c/c_1 reduction

The experimental technique was the same as that given in the legends to Figs. 2 and 3, the cells being incubated in the presence of 20 mg of albumin/ml containing endogenous fatty acids; $7.5 \mu\text{M}$ -A23187, 14 mM-ethanol, 2.5 nM-valinomycin (VAL), 0.5 mM-amytal, 0.1 μM -glucagon (GLUC) or 20 μM -phenylephrine (PHEN) was added as indicated. In trace (b) (i) the large increase in fluorescence caused by the inherent fluorescence of A23187 was subtracted, and the sensitivity of the fluorimeter was only 30% of that used in other experiments. The β -hydroxybutyrate/acetoacetate ratio was 1.72 in control cells, rising to 4.41 10 min after addition of 14 mM-ethanol, and to 1.97 10 min after glucagon addition. The corresponding lactate/pyruvate ratios were 28.7 (control), 209 (ethanol) and 30.1 (glucagon) and the rates of gluconeogenesis (nmol/min per mg of protein) were 6.3 (control), 2.6 (ethanol) and 8.1 (glucagon). The increase in O_2 consumption was 12.5% with glucagon treatment, but was decreased by 10% with ethanol. The effects of amytal addition are detailed in Table 3.

cytes incubated in the presence of fatty acids (results not shown). Thus cytochrome c/c_1 was more reduced (15% as opposed to 12%), and the increase in reduction caused by Ca^{2+} was greater, whereas the increase in NAD(P)H was smaller but less transient.

Effects of amytal and pent-4-enoate on the hormonal activation of the respiratory chain and gluconeogenesis from L-lactate

Amytal inhibits the respiratory chain within the NADH: ubiquinone oxidoreductase (Ernster *et al.*, 1955; Horgan *et al.*, 1968; Palmer *et al.*, 1968). In Fig. 5 and Table 3 we demonstrate that addition of 0.5 mM-amytal to hepatocytes causes an oxidation of cytochrome c/c_1 that almost exactly reverses the reduction caused by hormones. This concentration of amytal reversed the effects of glucagon on the rates of gluconeogenesis and O_2 consumption (Table 3). Data are also given for the β -hydroxybutyrate/acetoacetate and lactate/pyruvate ratios to estimate the mitochondrial and cytoplasmic NADH/NAD $^{+}$ ratios respectively. These ratios are raised only slightly by the presence of the inhibitor. This concentration of amytal quenched the fluorescence of NAD(P)H, and therefore the expected increase in this parameter could not be observed. The decrease in the rate of gluconeogenesis caused by 0.5 mM-amytal was not

associated with a substantial fall in tissue ATP content (Table 3, and Thomas & Halestrap, 1981). Thus it is unlikely that it is a decrease in [ATP] which inhibits gluconeogenesis. This is shown more clearly by the data of Fig. 7, where the effects of the uncoupler 2,4-dinitrophenol on the rate of gluconeogenesis and the tissue ATP content are compared with those of amytal. A 30% inhibition of gluconeogenesis was associated with about a 25% decrease in tissue ATP content when induced by dinitrophenol, whereas only about a 7% decrease in ATP was observed when inhibition was induced by amytal. It is also significant that the sensitivity of gluconeogenesis from L-lactate to inhibition by α -cyano-4-hydroxycinnamate is altered by 0.5 mM-amytal in a manner exactly opposite to the effect of gluconeogenesis hormones on this parameter (Thomas & Halestrap, 1981). Other, more powerful, inhibitors of the bc_1 complex, such as antimycin and 2-heptyl-4-hydroxyquinoline *N*-oxide, proved to be unsuitable for these studies, since it was very difficult to control the inhibitor concentration in the range that gave only partial inhibition of gluconeogenesis. This was for three reasons. Firstly, the inhibitors bound to the albumin present; secondly, the sigmoid shape of the concentration-dependence of their inhibition of the respiratory chain (see Nijs, 1967; Thayer & Rubin, 1982); thirdly, the

Fig. 6. Comparison of the effects of hormones, Ca^{2+} and valinomycin on the time course of NAD(P)H fluorescence, cytochrome c/c_1 reduction and light-scattering by hepatocytes and isolated liver mitochondria

In (a) fluorescence (continuous line) and cytochrome c/c_1 reduction (dots) were measured in the presence of 2.5 mM- Ca^{2+} and in the absence of fatty acids as described in Figs. 2 and 3. The light-scattering data (broken line) was obtained as described by Quinlan *et al.* (1983). Abbreviations: VASO, vasopressin; VAL, valinomycin. In (b) isolated liver mitochondria were incubated at 4 mg of protein/ml and 37 °C in buffer containing 125 mM-KCl, 10 mM-Mops, 7 mM-Tris base, 0.5 mM-EGTA, 2.5 mM- MgCl_2 , 2.5 mM-potassium phosphate, 10 mM-glucose, 0.25 unit of hexokinase/ml, 5 mg of defatted albumin/ml, 5 mM-sodium L-glutamate and 1 mM-malate adjusted to pH 7.2. Fluorescence (broken lines), cytochrome c/c_1 reduction state (dots) and light-scattering were measured in parallel experiments on the same batch of mitochondria. In (b) (ii) cytochrome b reduction ($A_{562-575}$) was also measured. All traces begin 6 min after mitochondria were added to the medium, to allow parameters to reach a steady state (see the text). Further details of the experimental technique are given in Halestrap *et al.* (1986) and the Experimental section of the present paper. Additions were made as indicated: 0.4 mM-ADP, 0.37 mM Ca^{2+} , 1 μM carbonyl cyanide p -trifluoromethoxyphenylhydrazone (FCCP), 1 nM-valinomycin (VAL) and 1 $\mu\text{g/ml}$ antimycin (ANTI).

inhibitors were metabolized by the liver cells during the course of the incubation (P. J. Quinlan, unpublished work).

In liver cells from starved animals, the major respiratory fuel is fatty acids, since pyruvate dehydrogenase is highly inactive and fatty acids are abundant (Wieland, 1983). Thus it is of interest to see whether the inhibitor of fatty acid oxidation, pent-4-enoate (Sherratt, 1981), has the same effect as respiratory-chain inhibitors on the hormonal stimulation of gluconeogenesis. In Figs. 2 and 3 and Table 1 we include data on the effects of 1 mM-pent-4-enoate on the increases in NAD(P)H fluorescence and cytochrome c/c_1 reduction induced by hormones. It is apparent that both changes are greatly decreased by the presence of pent-4-enoate, as is the

stimulation of gluconeogenesis. Thus these data imply that an increase in fatty acid oxidation does contribute towards the increase in NAD(P)H fluorescence and cytochrome c/c_1 reduction and is important in the stimulation of gluconeogenesis.

DISCUSSION

Mechanisms involved in the hormonal control of respiration in hepatocytes

As outlined in the Introduction section, the accepted means of controlling electron flow from NADH to cytochrome c are the NADH/NAD $^{+}$ ratio or the protonmotive force. Our data clearly show that the former mechanism alone cannot account for the changes in O_2 consumption and cytochrome c/c_1 reduction. The protonmotive force is reflected by the intramitochondrial ATP/ADP ratio. However, hormone treatment of liver cells or the perfused liver has not been shown to change either of these parameters in the required direction (see the Introduction section). Thus, in agreement with Bucher & Sies (1980), we would conclude that under conditions of active gluconeogenesis the relationship of the redox state of cytochrome c to the ratios of NADH/NAD $^{+}$ and ATP/ADP is not one of thermodynamic equilibrium, as suggested by Erecinska & Wilson (1982). Rather our data suggest that the rate of respiration under metabolic conditions follows the reduction state of cytochrome c , which itself may be determined by a mechanism independent of the protonmotive force or NADH/NAD $^{+}$ ratio.

The very rapid rise in NAD(P)H fluorescence after hormone addition has the same time course as the rise in cytoplasmic $[\text{Ca}^{2+}]$ detected with Quin 2 (Charest *et al.*, 1983; Thomas *et al.*, 1985) and could be explained by activation of the intramitochondrial dehydrogenases known to occur in liver mitochondria in response to hormones (McCormack, 1985a,b). There is also good circumstantial evidence for this from the study of substrate oxidation and pyruvate dehydrogenase activation in intact hepatocytes and perfused livers (Sugden *et al.*, 1980; Taylor *et al.*, 1983; Sugden & Watts, 1983; Haussinger & Sies, 1984; Oviyasu & Whitton, 1984; Staddon & McGivan, 1984, 1985) as well as by more direct measurements of mitochondrial $[\text{Ca}^{2+}]$ (Berthon *et al.*, 1981; Shears & Kirk, 1984; Kleineke & Soling, 1985; McCormack, 1985a,b). The subsequent decline in NAD(P)H fluorescence could be due to the raised intramitochondrial $[\text{Ca}^{2+}]$ causing swelling of the mitochondria (Quinlan *et al.*, 1983; Halestrap *et al.*, 1986) and consequent stimulation of electron flow from NADH to cytochrome c (Halestrap, 1982; Armston *et al.*, 1982). The ability of valinomycin to cause oxidation of NADH at the same time as cytochrome c/c_1 becomes reduced is explained by the volume-dependent increase in respiratory-chain activity occurring in the absence of an effect of Ca^{2+} on the mitochondrial dehydrogenases. We have provided evidence for the involvement of Ca^{2+} in this increase in matrix volume (Quinlan *et al.*, 1983; Halestrap *et al.*, 1986). In the Ca^{2+} -free experiments recorded in Figs. 2 and 3, conditions under which swelling induced by phenylephrine and vasopressin is prevented and that by glucagon is decreased (Quinlan *et al.*, 1983), the increase in cytochrome c/c_1 reduction becomes transient, just as is the NAD(P)H fluorescence. This is consistent with the maintenance of the increased

Table 3. Effect of amytal and glucagon on gluconeogenesis, respiration, cellular ATP content and the intracellular redox state

Hepatocytes (4 mg of cell protein/ml) were incubated at 37 °C with 10 mM-lactate, 1mM-pyruvate and albumin (2%, w/v) containing endogenous fatty acids as described in the Experimental section. Hormone additions were made after preincubation of cells for 15 min, and amytal was added 5 min later. The incubations were continued for a further 15 min before termination with HClO₄ (2%, w/v). After the precipitated protein was sedimented by centrifugation, samples of the supernatant were assayed for metabolites as outlined in the Experimental section. The rate of gluconeogenesis was estimated over a 15 min period after the cells had been incubated for 20 min. Rates of O₂ utilization were measured in parallel incubations in the same batch of cells. Control values are means $\pm$ S.E.M. of data from eight cell preparations, with each experimental value being the mean obtained from four separate cell incubations. Experimental values are given as the difference between experimental and control values, expressed as a percentage of the control value. Changes in cytochrome *c/c*₁ were determined as described in Fig. 5 and are given as means $\pm$ S.E.M. of observations on five cell preparations. The statistical significance of differences from the control means was calculated by a paired *t* test. ****P* < 0.001; ***P* < 0.01; **P* < 0.05.

	Control value	Difference between experimental and control values (%)		
		Control + 0.5 mM-amytal	Glucagon (100 nM)	Glucagon + 0.5 mM-amytal
Rate of glucose production (nmol/min per of mg protein)	6.76 $\pm$ 0.23	-9.5 $\pm$ 2.5**	25.4 $\pm$ 2.5***	4.8 $\pm$ 2.7
Rate of O ₂ consumption (ng-atoms of O/min per mg of protein)	53.3 $\pm$ 3.0	-8.5 $\pm$ 0.99***	12.5 $\pm$ 0.8***	1.5 $\pm$ 0.7
Cell ATP content (nmol/mg of protein)	7.66 $\pm$ 0.43	-2.6 $\pm$ 1.9	4.8 $\pm$ 1.4**	-4.8 $\pm$ 1.5*
Lactate/pyruvate ratio	28.7 $\pm$ 2.8	9.4 $\pm$ 3.1*	5.1 $\pm$ 1.3**	16.3 $\pm$ 3.9**
3-Hydroxybutyrate/acetoacetate ratio	1.72 $\pm$ 0.17	17.0 $\pm$ 1.8***	14.1 $\pm$ 3.1**	36.3 $\pm$ 5.8***
Cytochrome <i>c/c</i> ₁ reduction (%)	18%	-29.6 $\pm$ 3.3***	26.4 $\pm$ 5.3**	-6.6 $\pm$ 6.7

Fig. 7. Comparison of the effects of amytal and 2,4-dinitrophenol on the rate of gluconeogenesis and the tissue ATP content

Hepatocytes were incubated as described in the legend to Table 3 with increasing concentrations (0.15–2.5 mM) of amytal (—) or 2,4-dinitrophenol (---), and measurements were made of the glucose produced and the tissue ATP content. Values are given as means $\pm$ S.E.M. (error bars) for at least four observations on different cell preparations. Where no error bars are given, the value is the mean of two experiments.

cytochrome *c/c*₁ reduction state being a consequence of the Ca²⁺-mediated increase in mitochondrial volume. The effects of A23187 to increase cytochrome *c/c*₁ reduction as well as NAD(P)H fluorescence can be accounted for

by the ability of A23187 to increase mitochondrial volume (Halestrap *et al.*, 1986) as well as to stimulate the mitochondrial dehydrogenases.

Further evidence for a biphasic mechanism for the hormonal stimulation of respiration is seen in experiments on the perfused liver. Addition of glucagon or phenylephrine causes a biphasic increase in O₂ consumption, consisting of a very rapid increase corresponding to the rapid increase in NAD(P)H fluorescence, followed by a slight decline and then a slow increase again (Williamson *et al.*, 1969b; Buxton *et al.*, 1982; Sies *et al.*, 1983; Kimura *et al.*, 1984). This is entirely consistent with a rapid Ca²⁺-dependent activation of mitochondrial dehydrogenases giving the initial burst of O₂ consumption, followed by the effects of the increase in matrix volume to give the slower phase of increased O₂ uptake. LaNoue *et al.* (1984) have shown that duroquinol oxidation by hepatocytes is not stimulated by hormone treatment and have concluded that direct stimulation of the respiratory chain does not occur *in vivo*. We have confirmed their data, but have shown (A. P. Halestrap, unpublished work) that uncoupled duroquinol oxidation by isolated mitochondria is insensitive to changes in the mitochondrial volume and would not therefore be expected to be stimulated by hormone treatment of cells. Mitochondria isolated very rapidly from liver cells by sonication (Quinlan *et al.*, 1983) or in crude liver homogenates (Jensen *et al.*, 1983) have been shown to exhibit a hormonal stimulation of respiration, which supports such stimulation occurring *in vivo*. However, activation of the respiratory chain by glucagon could also occur by a mechanism other than the increase in volume, since mitochondria isolated from rats treated with glucagon show enhanced respiratory-chain activity in the absence

of any increase in matrix volume (Whipps & Halestrap, 1984). It remains to be established whether these effects are caused by the same mechanism as operates *in situ* or are an artefact of mitochondrial isolation dependent on the activity of phospholipase A₂ during mitochondrial isolation (Armstrong & Halestrap, 1984).

Importance of fatty acid oxidation for the hormonal stimulation of respiration

The volume-mediated stimulation of respiration may be related to the particular sensitivity of fatty acid oxidation to small increases in the matrix volume (Osmundsen & Bremer, 1976; Otto & Ontko, 1982; J. Dunlop & A. P. Halestrap, unpublished work). Indeed, such a mechanism may also account for the stimulatory effect of α -agonists and vasopressin on fatty acid oxidation (Sugden & Watts, 1983), where effects on carnitine palmitoyltransferase I via changes in malonyl-CoA concentration are thought not to occur. It should also be noted that the present experiments are performed in cells from starved animals, where malonyl-CoA inhibition of the transferase may not be very effective anyway (see Saggerson, 1982).

The data of Fig. 1 and Table 1 provide further evidence for the importance of a stimulation of fatty acid oxidation, since the increase in cytochrome *c/c*₁ reduction is greater in the presence than in the absence of fatty acids and is greatly decreased by the presence of 1 mM-pent-4-enoate. Williamson *et al.* (1970) have also studied the effects of pent-4-enoate on the metabolism of the perfused liver and found that the inhibition of gluconeogenesis and ketogenesis caused by it correlated with the inhibition of fatty acid oxidation and decrease in tissue acetyl-CoA. Conversely, the stimulation of gluconeogenesis and ketogenesis caused by oleate addition correlated with an increase in fatty acid oxidation and tissue acetyl-CoA (Williamson *et al.*, 1969*a,b,c*). Furthermore Williamson *et al.* (1969*b*) have demonstrated that the increase, in O₂ consumption and NAD(P)H fluorescence become transient when fatty acid oxidation is inhibited by decanoylcarnitine.

The ability of glucagon to increase fatty acid oxidation and so to increase mitochondrial acetyl-CoA concentrations and pyruvate carboxylase activity was originally considered to be the means by which the hormone stimulated gluconeogenesis from L-lactate (Struck *et al.*, 1965, 1966; Williamson *et al.*, 1966*a,b*; Soling *et al.*, 1968; Wieland *et al.*, 1969). However, the observations that the effects of glucagon and oleate on gluconeogenesis were additive (Ross *et al.*, 1967*a*; Exton *et al.*, 1969; Frohlich & Wieland, 1971, 1972), the inability of the inhibitor of hepatic lipolysis, glycodiazine, to inhibit the effect of glucagon (Menahan & Wieland, 1969; Frohlich & Wieland, 1971), and the distinctive effects of glucagon and fatty acids on the compartmentation of cellular metabolites (Brooks *et al.*, 1980), have led to the conclusion that the stimulation of gluconeogenesis is not secondary to the stimulation of fatty acid oxidation. The data of Table 1 confirm that the effects of hormones on gluconeogenesis can still be observed in the presence of 1 mM-oleate. Indeed there is an almost identical increase in the rate of glucose production seen on addition of hormone in the presence or absence of oleate, although the absolute rate of gluconeogenesis more than doubles. However, it is not logical to conclude from these data and the other observations mentioned above that the effects

of hormones to increase gluconeogenesis cannot be mediated, at least in part, through an increase in fatty acid oxidation. If the stimulation of fatty acid oxidation is intramitochondrial at the level of the respiratory chain rather than through the supply of fatty acids, it would be expected that the effects of fatty acids and hormones could be additive, as observed.

Role of the stimulation of the respiratory chain in the regulation of gluconeogenesis from L-lactate

The ability of 0.5 mM-amytal to reverse the effects of hormones on gluconeogenesis, respiration and cytochrome *c/c*₁ reduction described in Table 3 and Fig. 5 strongly support a role for stimulation of the respiratory chain in the regulation of gluconeogenesis. So do the effects of pent-4-enoate. It is also of interest that the most effective hypoglycaemic agents are either mild respiratory-chain inhibitors or fatty-acid-oxidation inhibitors (Sherratt, 1981). If it is assumed that 1 mg of cell protein represents 0.34 mg of mitochondrial protein (Quinlan *et al.*, 1983), then the rate of O₂ uptake seen in starved cells in the presence of 1 mM-oleate is equivalent to about 150 ng-atoms of O/min per mg of mitochondrial protein. This rate of respiration is faster than the rate of palmitoylcarnitine oxidation in State 3 seen in isolated mitochondria at 37 °C, similar to the rate of succinate oxidation in State 3 and about 70% of the maximal uncoupled rate (Halestrap, 1978). Addition of 0.7 mM-2,4-dinitrophenol to uncouple maximally hepatocytes incubated in the presence of 1 mM-oleate gave a 162 ± 10% stimulation of respiration (mean ± S.E.M. of four observations on different cell preparations). The higher uncoupled rates of O₂ consumption seen in mitochondria *in situ* than in isolated mitochondria have also been reported by LaNoue *et al.* (1984). These observations suggest that the rate of electron flow through the respiratory chain under the conditions of rapid gluconeogenesis in the presence of fatty acids is about 40% of the maximal uncoupled rate. This is consistent with the rate of respiration being a limiting factor in the rate of gluconeogenesis and a suitable site for hormonal control, as we have previously suggested (Halestrap, 1981; Halestrap *et al.*, 1983). This is further supported by a consistent observation that the rate of O₂ consumption decreased progressively as the O₂ content of the medium fell below 300 ng-atoms/ml (results not shown). Under these conditions it is known that the rates of gluconeogenesis also decrease (Wolfe *et al.*, 1983).

The increased O₂ consumption, NAD(P)H fluorescence and cytochrome *c/c*₁ reduction begin within 1 s of hormone addition and probably precede the increase in gluconeogenesis by several seconds (Taylor *et al.*, 1983), which corresponds more directly to the increase in mitochondrial volume (Quinlan *et al.*, 1983). Increased gluconeogenesis will require increased supply of ATP and hence increased respiration. The two conventional ways by which this could be achieved are by increasing the NADH/NAD⁺ or decreasing the ATP/ADP ratios (Erecinska & Wilson, 1982). However, both a decrease in ATP/ADP and an increase in NADH/NAD⁺ ratios might inhibit gluconeogenesis (see, e.g., Fig. 2, and Berry *et al.*, 1983*a,b*; Sistare & Haynes, 1985), which is exactly the opposite of what is required. If, however, a mechanism exists to stimulate the respiratory chain directly, it is possible to have faster rates of ATP synthesis and O₂ consumption at the same ATP/ADP and

NADH/NAD⁺ ratios. The increase in mitochondrial volume may provide such a mechanism and in addition cause a selective increase in fatty acid oxidation, which has the added advantage of providing acetyl-CoA for the stimulation of pyruvate carboxylase. In addition, through their stimulation of 2-oxoglutarate dehydrogenase these hormones cause a decrease in the concentration of glutamate (Siess *et al.*, 1977, 1978; Haussinger & Sies, 1984; Staddon & McGivan, 1984, 1985) which is an inhibitor of pyruvate carboxylase (Scrutton & White, 1974). We have demonstrated previously that stimulation of the respiratory chain is a key event in the stimulation of pyruvate carboxylation in mitochondria isolated from glucagon-treated rats (Halestrap, 1978; Halestrap & Armston, 1984). The present paper clearly shows that such stimulation of the respiratory chain is a key event in the hormonal stimulation of gluconeogenesis in isolated rat hepatocytes.

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