Biochem. J. (1987) 247, 449–457 (Printed in Great Britain)

Evidence that the flux control coefficient of the respiratory chain is high during gluconeogenesis from lactate in hepatocytes from starved rats

Implications for the hormonal control of gluconeogenesis and action of hypoglycaemic agents

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1. Increasing concentrations of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), a mild respiratory-chain inhibitor [Halestrap (1987) Biochim. Biophys. Acta **927**, 280–290], caused progressive inhibition of glucose production from lactate + pyruvate by hepatocytes from starved rats incubated in the presence or absence of oleate and gluconeogenic hormones. 2. No significant changes in tissue ATP content were observed, but there were concomitant decreases in ketone-body output and cytochrome *c* reduction and increases in NADH fluorescence and the ratios of [lactate]/[pyruvate] and [β-hydroxybutyrate]/[acetoacetate]. 3. The inhibition by DCMU of palmitoylcarnitine oxidation by isolated liver mitochondria was used to calculate a flux control coefficient of the respiratory chain towards gluconeogenesis. In the presence of 1 mM-oleate, the calculated values were 0.61, 0.39 and 0.25 in the absence of hormone and in the presence of glucagon or phenylephrine respectively, consistent with activation of the respiratory chain *in situ* as previously suggested [Quinlan & Halestrap (1986) Biochem. J. **236**, 789–800]. 4. Cytoplasmic oxaloacetate concentrations were shown to decrease under these conditions, implying inhibition of pyruvate carboxylase. 5. Inhibition of gluconeogenesis from fructose and dihydroxyacetone was also observed with DCMU and was accompanied by an increased output of lactate + pyruvate, suggesting that activation of pyruvate kinase was occurring. With the latter substrate, measurements of tissue ADP and ATP contents showed that DCMU caused a small fall in [ATP]/[ADP] ratio. 6. Two inhibitors of fatty acid oxidation, pent-4-enoate and 2-tetradecylglycidate, were shown to abolish and to decrease respectively the effects of hormones, but not valinomycin, on gluconeogenesis from lactate + pyruvate, without changing tissue ATP content. 7. It is concluded that the hormonal increase in mitochondrial matrix volume stimulates fatty acid oxidation and respiratory-chain activity, allowing stimulation of pyruvate carboxylation and thus gluconeogenesis to occur without major changes in [ATP]/[ADP] or [NADH]/[NAD^+] ratios. 8. The high flux control coefficient of the respiratory chain towards gluconeogenesis may account for the hypoglycaemic effect of mild respiratory-chain inhibitors.

INTRODUCTION

Hormones which stimulate gluconeogenesis by isolated rat hepatocytes also increase the rate of O_2 consumption, but without an increase either in [ADP]/[ATP] ratio or, in many cases, in [NADH]/[NAD^+] ratio (see Quinlan & Halestrap, 1986, and references therein). We have shown that reversing the effects of hormones on respiration by addition of the mild respiratory-chain inhibitor amytal also reverses the stimulation of gluconeogenesis (Quinlan & Halestrap, 1986), and have suggested that activation of the respiratory chain plays an important role in the physiological stimulation of gluconeogenesis. We have demonstrated that gluconeogenic hormones are able to stimulate the respiratory chain by increasing the mitochondrial volume in a Ca^{2+}-dependent manner (Quinlan *et al.*, 1983; Halestrap *et al.*, 1986; Quinlan & Halestrap, 1986). Such an increase in matrix volume gives some stimulation of electron flow from Complex I and Complex II of the respiratory chain into Complex III (Armston *et al.*, 1982; Halestrap, 1982) and has a very large stimulatory effect on fatty acid oxidation by enhancing electron flow from the electron-transferring flavoprotein to ubiquinone (Halestrap & Dunlop, 1986). In isolated mitochondria we have shown that the rate of pyruvate carboxylation may be limited by the activity of the respiratory chain (Halestrap & Armston, 1984), and it seems likely that pyruvate carboxylase is the step at which gluconeogenesis is inhibited by respiratory-chain inhibitors in hepatocytes. Certainly pyruvate carboxylase is the enzyme with the highest flux control coefficient for gluconeogenesis from L-lactate + pyruvate in hepatocytes from starved rats (Groen *et al.*, 1986).

In the present paper we seek to quantify the regulatory potential of the respiratory chain towards gluconeogenesis by using the mild respiratory-chain inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), which inhibits the respiratory chain at the same sites as are activated by treatment of rats with gluconeogenic hormones (Halestrap, 1987). In addition, we attempt to establish the nature of the regulatory link between the respiratory chain and the gluconeogenic pathway and the relationship this has with the hormonal stimulation of gluconeogenesis and fatty acid oxidation.

Abbreviations used: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; TDGA, 2-tetradecylglycidate.

EXPERIMENTAL

Materials

Except where stated below, the sources of all chemicals and biochemicals were as given in Quinlan & Halestrap (1986) and references cited therein. DCMU was a gift from DuPont, Stevenage, Herts., U.K., and was recrystallized once from ethanol before use, and 2-tetradecylglycidate (TDGA) was given by McNeil Pharmaceuticals, Spring House, PA, U.S.A. Both reagents were dissolved in dimethyl sulphoxide at suitable concentrations to allow final concentrations in the incubation media to be achieved by addition of less than 2 μl of stock reagent/ml. Solvent alone was shown to have no effects on any of the parameters measured. Isolated rat liver mitochondria and hepatocytes were prepared from 24 h-starved male Wistar rats (300 g body wt.) as described previously (Halestrap, 1978; Quinlan *et al.*, 1983). Defatted bovine serum albumin (5 mg/ml) was added to the initial homogenization medium used for the preparation of mitochondria.

Incubation of isolated liver cells and mitochondria

Liver cells were incubated in bicarbonate-buffered saline (Krebs & Henseleit, 1932) containing 20 mg of defatted and dialysed bovine serum albumin/ml and equilibrated with an atmosphere of O_2/CO_2 (19:1) at 37 °C. Additions of substrates, inhibitors and hormones were made as indicated in the legends to the Figures and Tables. Unless intracellular metabolites were to be measured, the cell protein concentration was 4 mg/ml, and samples of the cell suspension were taken for metabolite assay after 30 and 45 min incubation in a shaking water bath as described previously (Thomas & Halestrap, 1981; Quinlan & Halestrap, 1986). For measurement of intracellular metabolites, the cell concentration was 10 mg of protein/ml and samples were taken after 30 and 45 min for glucose assay as above. In addition, at the latter time two 0.5 ml samples of cell suspension were rapidly layered on top of 0.25 ml of oil (silicone oil MS550/dinonyl phthalate, 2:1, v/v) covering 50 μl of $HClO_4$ [20% (w/v) and containing 25% (w/v) glycerol]. Cells were then sedimented through the oil by centrifugation at 9000 ***g*** for 1 min, and the supernatant was removed and deproteinized with 2% $HClO_4$. After removal of the oil, the two pellet extracts were vortex-mixed and centrifuged (10000 ***g***) to remove protein. Both acidified supernatants were combined and neutralized with 5 M-KOH containing 0.5 M-Mops.

Isolated mitochondria were incubated at 37 °C in a thermostatically controlled Clark-type oxygen-electrode chamber at a protein concentration of 1.5 mg/ml in medium containing 125 mM-KCl, 10 mM-Mops, 7 mM-Tris, 2.5 mM-potassium phosphate, 2.5 mM-$MgCl_2$, 0.5 mM-EGTA, 5 mg of defatted albumin/ml and other additions as indicated.

Assay of metabolites

Glucose, pyruvate, lactate, β-hydroxybutyrate, malate and acetoacetate in the neutralized extracts were assayed spectrophotometrically by using standard enzymic assays as referenced elsewhere (Quinlan & Halestrap, 1986; Groen *et al.*, 1983). ATP and ADP were assayed in the neutralized pellet extract by the methods of Lamprecht & Trautschold (1974) and Jaworek *et al.* (1974), whereas in other experiments luciferase was used to assay ATP in the deproteinized total cell suspension (see Quinlan & Halestrap, 1986). The ATP concentrations determined by both techniques were essentially the same and similar to values reported previously from this laboratory (Thomas & Halestrap, 1981; Quinlan & Halestrap, 1986). Cytosolic oxaloacetate concentrations were estimated from the [lactate]/[pyruvate] ratio in the supernatant and the malate in the cell pellet extract as described by Groen *et al.* (1983). No attempts were made to correct for compartmentation of cellular malate. Protein was measured by a modified biuret method (Gornall *et al.*, 1949), with bovine serum albumin as standard.

Measurement of NADH fluorescence and cytochrome *c* reduction state of liver cells

This was performed by using a home-built computerized spectrophotometer/fluorimeter exactly as described previously (Quinlan & Halestrap, 1986).

RESULTS AND DISCUSSION

Effects of increasing DCMU concentrations on gluconeogenesis, ketone-body output and cellular ATP content

In Fig. 1 we present data on the effects of increasing the concentration of DCMU on the ATP content of isolated rat liver cells and on their rate of gluconeogenesis from 10 mM-L-lactate + 1 mM-pyruvate. Experiments were performed in both the presence (Fig. 1*a*) and the absence (Fig. 1*b*) of 1 mM-sodium oleate. Parallel measurements of ketone-body output were made when oleate was present, and the results are recorded in Fig. 2, where data are also presented for the [β-hydroxybutyrate]/[acetoacetate] ratio. In addition, the influence of 0.1 μM-glucagon and 20 μM-phenylephrine on the sensitivity of all parameters to DCMU was investigated. In all cases the mean control parameter value (no hormone, no DCMU) is given in the legend, and all other values are expressed as a percentage of this control value to allow data from different experiments to be combined.

As the concentration of DCMU was increased, there was a progressive inhibition of gluconeogenesis whether or not oleate was present. The cellular ATP content showed no significant decrease; indeed, at 0.1 mM-DCMU a slight increase in ATP was apparent, especially in the presence of oleate and glucagon. Only at concentrations of DCMU above 0.5 mM was there any evidence for a decrease in ATP, and at these concentrations there is evidence that DCMU acts as a very weak uncoupler of oxidative phosphorylation (Halestrap, 1987). Coincident with the decrease in gluconeogenesis, there was a small decrease in the output of ketone bodies, to about 75% of the control value at 0.5 mM-DCMU, and thereafter falling no further. The plateau in the effects of DCMU at concentrations above 0.5 mM on ketone-body output corresponds to the small decrease in ATP noted above. It could reflect a balance between the inhibition of fatty acid oxidation by a direct interaction of DCMU with the respiratory chain and an increase in electron flow as a result of the uncoupling effect of the inhibitor.

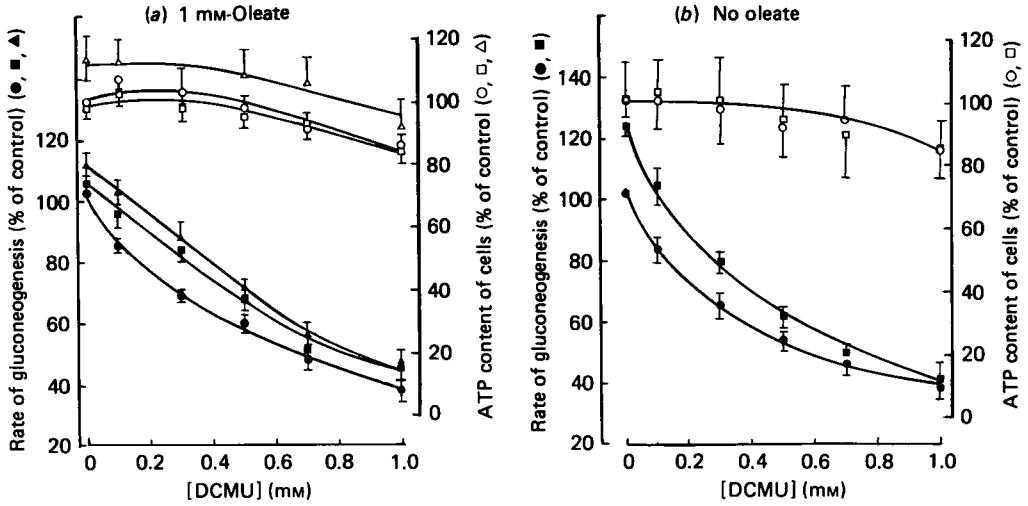

Fig. 1. Effects of increasing concentrations of DCMU on gluconeogenesis from lactate and pyruvate and on the ATP content of hepatocytes

Hepatocytes from starved rats were incubated as described in the Experimental section with 10 mM-L-lactate, 1 mM-pyruvate and 20 mg of defatted bovine serum albumin/ml in the presence (*a*) or absence (*b*) of 1 mM-sodium oleate and DCMU at the concentration shown. After 20 min preincubation, hormones were added as appropriate (●, ○, none; ■, □, 0.1 μM-glucagon; ▲, △, 20 μM-phenylephrine) and the rates of gluconeogenesis (●, ■, ▲) measured between 30 min and 45 min. ATP was measured in the deproteinized cell suspension at 45 min by luciferase assay (○, □, △). All values are expressed as the percentage of the value in the absence of hormone and DCMU, as means ± S.E.M. (error bars) for nine separate experiments (different cell preparations) for control and glucagon incubations in the presence of oleate and five under the remaining conditions. The mean control values (± S.E.M.) for ATP were 7.77 ± 0.79 and 7.89 ± 1.21 nmol/mg of protein in the presence and absence of oleate respectively, and for the rate of gluconeogenesis the corresponding values were 7.55 ± 0.63 and 4.18 ± 0.26 nmol/min per mg of protein.

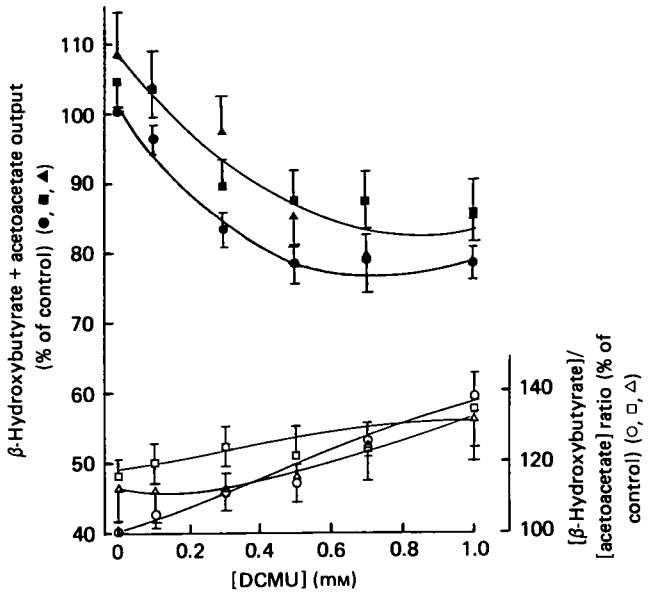

Fig. 2. Effects of increasing concentrations of DCMU on the output of ketone bodies by hepatocytes and the [β-hydroxybutyrate]/[acetoacetate] ratio

Data were obtained from the same experiments as those in Fig. 1(*a*). The control output of ketone bodies (acetoacetate + β-hydroxybutyrate) after incubation for 45 min was 224 ± 29 nmol/mg of protein and the [β-hydroxybutyrate]/[acetoacetate] ratio 1.35 ± 0.10.

Flux control coefficient of the respiratory chain towards gluconeogenesis

In order to quantify the regulatory potential of the individual enzymes of gluconeogenesis, Groen *et al.* (1983, 1986) have attempted to measure the flux control coefficients of each step. This parameter, sometimes also known as the control strength, is defined as $(\partial J/J_0)/(\partial V/V_0)$ where J_0 is the flux through the pathway at equilibrium and V_0 corresponds to the forward velocity of the enzyme in question. ∂J is the perturbation in J caused by an extremely small perturbation (∂V) in V (see Westerhoff *et al.*, 1984). One way of determining flux control coefficients is to measure the change in the flux through a pathway caused by increasing concentrations of a specific inhibitor of the enzyme of interest. A plot of the initial slope of the inhibition of pathway flux against [inhibitor] can then be used to calculate $\partial J/J_0$. $\partial V/V_0$ may be calculated from the known kinetic parameters of that enzyme, or, for a complex system like the respiratory chain, it must be determined experimentally as outlined below.

We have attempted to use DCMU in this way in order to calculate an overall flux control coefficient of the respiratory chain towards gluconeogenesis. Under the conditions employed in the present experiments, fatty acids are the major respiratory fuel, and it has been proposed that they are being oxidized at rates that approximate to the maximal rate of ADP-stimulated oxidation of palmitoylcarnitine by isolated liver mitochondria (Quinlan & Halestrap, 1986). Thus rates of

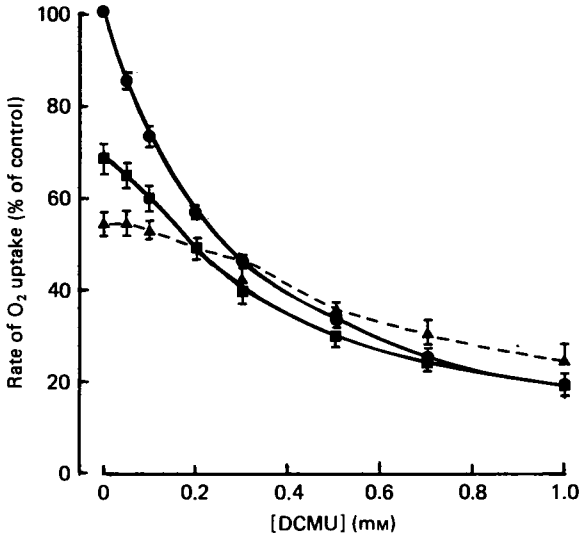

Fig. 3. Effects of increasing DCMU concentrations on the rate of oxidation of palmitoylcarnitine by isolated rat liver mitochondria

Rates of palmitoylcarnitine oxidation were measured at 37 °C in an oxygen electrode as described in the Experimental section. After preincubation for 1 min with 50 μM-palmitoylcarnitine, 0.5 mM-L-malate, 10 mM-glucose and DCMU at the required concentration, respiration was stimulated by addition of either 2 mM-ADP (●) or 1 mM-MgATP and sufficient hexokinase to give the required rate of O_2 consumption (■, ▲). Rates are expressed as a percentage of that in the absence of DCMU, as means ± S.E.M. (error bars) for four separate experiments using different mitochondrial preparations. The control rate was 123 ± 6 ng-atoms of O/min per mg of protein.

oxygen uptake are about 50 ng-atoms of O/min per mg of cell protein, which corresponds to a rate of about 130 ng-atoms of O/min per mg of mitochondrial protein (see also below). In Fig. 3 we show the effects of DCMU on the oxidation of palmitoylcarnitine by isolated liver mitochondria from starved male rats (of similar weight to those used for hepatocyte preparation) in the presence of ADP and at 37 °C. The buffer used had an osmolality of 300 mosmolal, which approximates to the physiological value. This is important, because the rate of fatty acid oxidation is extremely sensitive to the osmolality (Halestrap & Dunlop, 1986). Under these conditions the control rate of oxygen uptake was 123 ± 6 ng-atoms of O/min per mg of mitochondrial protein (mean ± S.E.M. for four separate mitochondrial preparations), and addition of DCMU caused a progressive inhibition of respiration similar to the effects observed previously for the oxidation of other NADH-generating substrates (Halestrap, 1987). The initial slope of this plot can be used to calculate $\partial V/V_0$. Our previous studies have shown that glucagon treatment did not alter the sensitivity of the respiratory chain towards inhibition by DCMU (Halestrap, 1987).

Under the conditions of Fig. 1, rates of glucose output were constant between 30 and 45 min of incubation, and thus flux through the gluconeogenic pathway was at equilibrium. From the data of Figs. 1(*a*) and 3 it appears that the initial slope of the plots of [DCMU] against the inhibition both of gluconeogenesis from lactate + pyruvate by hepatocytes and of State 3 oxidation of

Table 1. Flux control coefficients of the respiratory chain for gluconeogenesis from lactate + pyruvate under various conditions

Values were calculated from the ratio of the percentage inhibition of gluconeogenesis at 0.1 mM-DCMU (Fig. 1*a*) to the inhibition of palmitoylcarnitine oxidation at 0.1 mM-DCMU (Fig. 4) and are expressed as means ± S.E.M. for the numbers of observations shown in parentheses (n.d., not determined). Justification of this method of calculation is given in the text. Statistical significance of the differences between flux control coefficients in the presence and absence of hormones were calculated by paired Student's *t* test: $*P < 0.02$; $**P < 0.01$.

Hormone addition	Flux control coefficient	
	No oleate	1 mM-Oleate
None	0.86 ± 0.12 (5)	0.61 ± 0.10 (9)
0.1 μM-Glucagon	0.71 ± 0.15 (5)	0.39 ± 0.05** (9)
20 μM-Phenylephrine	n.d.	0.25 ± 0.07* (6)

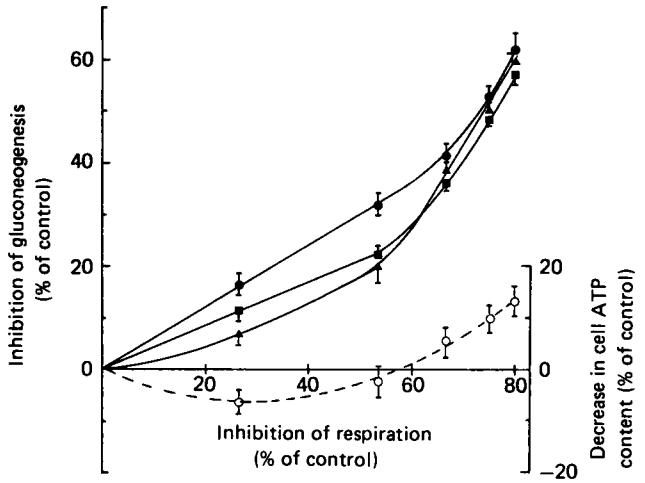

Fig. 4. Correlation of the effects of DCMU on the rates of respiration and gluconeogenesis

The relevant data are taken from Figs. 1 and 3. Rates of gluconeogenesis in the absence (●) and presence of glucagon (■) or phenylephrine (▲) and the ATP content of control cells (broken line, ○) are those determined in the presence of 1 mM-oleate

palmitoylcarnitine by isolated mitochondria may be calculated with reasonable accuracy from the inhibition observed at 0.1 mM-DCMU. The ratio of these values may then be used to calculate the flux control coefficient $[(\partial J/J_0)/(\partial V/V_0)]$. Values are given in Table 1 for various incubation conditions. An alternative approach is to plot the inhibition of the respiratory chain found at any DCMU concentration against the observed inhibition of gluconeogenesis and extrapolate this back to zero inhibition, at which point the slope of the graph is equivalent to the flux control coefficient. This is done in Fig. 4 for cells incubated with oleate in the presence and absence of glucagon and phenylephrine. In the absence of gluconeogenic hormones there appears to be a linear

relationship between the two parameters at concentrations of DCMU less than 0.5 mM, indicating that the flux control coefficient remains fairly constant over this range of respiratory-chain activity. This confirms the validity of using the values for inhibition of both parameters at 0.1 mM-DCMU to calculate the flux control coefficients in Table 1. Above 0.5 mM-DCMU the slope increases, indicating a higher flux control coefficient, and this corresponds to conditions where ATP concentrations are falling (broken line in Fig. 4). When either glucagon or phenylephrine was present, the initial slopes of the graph were less, as expected from the calculated values of the flux control coefficients in Table 1. However, in the presence of phenylephrine there is some evidence that the plot is not linear, even when inhibition is slight. This would lead to an overestimate of the flux control coefficient in Table 1.

In the absence of oleate, the flux control coefficients in the presence and absence of glucagon were again calculated from the inhibiton of both gluconeogenesis and palmitoylcarnitine oxidation at 0.1 mM-DCMU (Table 1). However, rates of respiration of cells in the absence of added oleate are only about half those in its presence (Quinlan & Halestrap, 1986), and therefore less than the maximal ADP-stimulated rate of respiration used to calculate $\partial V/V_0$. This lower rate of respiration could be the result of the decreased [NADH]/[NAD^+] ratio in the absence of fatty acids, or a higher [ATP]/[ADP] ratio insufficient to allow maximal State 3 rates of respiration. In Fig. 3 we demonstrate that, if rates of respiration are decreased by using glucose and hexokinase to control the [ATP]/[ADP] ratio, the initial sensitivity to inhibition by DCMU is greatly decreased. Furthermore, previous studies (Halestrap, 1987) have shown that the oxidation of other substrates showed a similar sensitivity to DCMU inhibition as does palmitoylcarnitine, except for succinate, which was considerably less sensitive. These considerations suggest that, if there are errors in the values of $\partial V/V_0$ used in the determination of the flux control coefficients, they are likely to lead to underestimation rather than overestimation of this value.

Effects of glucagon and phenylephrine on the flux control coefficient

The calculated flux control coefficient of the respiratory chain towards gluconeogenesis in control cells is high, and its 35–60% decrease after glucagon and phenylephrine treatment of liver cells in the presence of oleate (Table 1) is consistent with a hormonal stimulation of the respiratory chain. This substantiates previous evidence from our laboratory that hormones increase respiratory-chain activity *in vivo* both through a stimulation of electron flow and fatty acid β-oxidation mediated through an increase in matrix volume and through Ca^{2+}-dependent activation of mitochondrial dehydrogenases (Denton & McCormack, 1985; Quinlan & Halestrap, 1986; Halestrap & Dunlop, 1986; Halestrap, 1987). In the absence of fatty acids, glucagon decreased the flux control coefficient by about 15%, which was not statistically significant, but this was accompanied by a 25% or greater increase in gluconeogenesis. If this increase in gluconeogenesis was a result of a stimulation of the gluconeogenic pathway, increased ATP synthesis and therefore respiration would be required. In the absence of an increase in respiratory-chain activity, this would be expected to increase the flux control coefficient of the respiratory chain, in contrast with the small decrease actually observed.

An alternative explanation for a decrease in the flux control coefficient of the respiratory chain could be that the hormones inhibit pyruvate kinase. Since this step has a negative flux control coefficient towards gluconeogenesis, and the sum of the coefficients for a pathway must be 1, a less negative value of the coefficient for pyruvate kinase could be accompanied by a less positive value for the respiratory chain. However, a decrease in pyruvate kinase activity would be expected to produce an increase in the rate of gluconeogenesis, and this was not observed in the present experiments when 1 mM-oleate was present. This contrasts with the data of Table 4 and those of Quinlan & Halestrap (1986). Such variability in the ability of such hormones to stimulate gluconeogenesis in the presence of oleate can be seen in other studies, and references may be found elsewhere (Quinlan & Halestrap, 1986). A hormone effect was obtained in the absence of oleate in all experiments, as seen in Fig. 1 and Table 4, and under these conditions we cannot rule out the possibility that the effect of hormones on the flux control coefficient of the respiratory chain is secondary to an effect on pyruvate kinase. Nevertheless it would seem likely that, if the respiratory chain is stimulated in the presence of fatty acids, it is also stimulated in their absence. Thus our data support the conclusions of Rognstad & Katz (1977), Thomas & Halestrap (1981) and Patel & Olson (1986) that activation of pyruvate conversion into phosphoenolpyruvate is important in the stimulation of gluconeogenesis by glucagon, rather than those of Groen *et al.* (1983, 1986) and Sistare & Haynes (1985*a*), who suggest that inhibition of pyruvate kinase is more important.

Mechanism by which the respiratory chain interacts with gluconeogenesis

Role of pyruvate carboxylase. Under similar conditions to those in the experiments in the presence of oleate reported here, Groen *et al.* (1986) determined a flux control coefficient for pyruvate carboxylase of 0.83 and 0.56 in the presence and absence of glucagon respectively. In the presence of glucagon this enzyme appeared to be the only one exerting significant regulation of gluconeogenesis, whereas in the absence of hormone pyruvate kinase and other glycolytic enzymes had flux control coefficients suggesting moderate regulatory potential. Since changes in the activity of the respiratory chain can only influence gluconeogenesis through an indirect effect, the results of Groen *et al.* (1986) suggest that pyruvate carboxylase is the most likely locus of this effect. If this were the case, it would be expected that the intracellular oxaloacetate concentration would decrease in the presence of DCMU. This was investigated in the experiments reported in Table 2. Intracellular oxaloacetate concentrations were estimated by measurement of cellular malate concentrations and the [lactate]/[pyruvate] ratio of the incubation medium as described by Groen *et al.* (1983). When either alanine or lactate+pyruvate were substrates, the addition of 0.5 mM-DCMU caused a significant decrease in the calculated oxaloacetate concentration. This concentration of DCMU was used since it was the highest inhibitor concentration that was without effect on tissue ATP concentrations when lactate+pyruvate were the gluconeogenic substrates

Table 2. Effects of DCMU on ATP, ADP, malate and oxaloacetate concentrations in liver cells

Hepatocytes were incubated at about 10 mg of protein/ml in the presence of 20 mg of defatted albumin/ml, 1 mM-sodium oleate, the gluconeogenic substrates indicated and, when appropriate, 0.5 mM-DCMU. Glucose output was measured between 30 and 45 min. At 45 min 1 ml samples were transferred to small centrifuge tubes, and the cells were sedimented through oil into $HClO_4$ as described in the Experimental section. Lactate, pyruvate and glucose were assayed in the deproteinized supernatant, and ADP, ATP and malate in the $HClO_4$ pellet. Oxaloacetate was calculated from malate, pyruvate and lactate concentrations as described by Groen *et al.* (1983). Values are given as means ± S.E.M. for the numbers of experiments shown, each involving a different cell preparation. The statistical significance of the effects of 0.5 mM-DCMU was calculated by Student's paired *t* test: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Substrate	DCMU	No. of observations	Output into medium			Tissue content (nmol/mg of protein)			
			Glucose (nmol/min per mg of protein)	Lactate + pyruvate (nmol/45 min per mg of protein)	Lactate/ pyruvate ratio	ATP	ADP	Malate	Oxaloacetate ($\times 10^3$)
10 mM-Lactate + 1 mM-pyruvate	−	10	6.38 ± 0.40	−	12.59 ± 0.99	6.67 ± 0.53	−	2.65 ± 0.27	54.0 ± 6.3
	+	10	4.12 ± 0.31***	−	20.5 ± 3.03*	6.39 ± 0.61	−	2.93 ± 0.31	40.0 ± 5.3**
10 mM-Alanine	−	8	3.18 ± 0.26	25.2 ± 3.6	1.96 ± 0.29	7.38 ± 0.99	2.73 ± 0.42	0.57 ± 0.08	8.3 ± 1.3
	+	8	1.62 ± 0.22***	25.7 ± 3.6	3.68 ± 0.88*	6.27 ± 0.83**	3.27 ± 0.54***	0.68 ± 0.13	5.9 ± 1.2*
10 mM-Dihydroxy-acetone	−	8	9.80 ± 0.63	37.4 ± 5.9	3.49 ± 0.70	7.08 ± 0.59	2.85 ± 0.22	−	−
	+	8	6.52 ± 0.45***	80.1 ± 14.4**	4.69 ± 0.76	6.29 ± 0.54*	3.36 ± 0.23***	−	−

(Fig. 1*a*). It should be noted that the lactate + pyruvate output and the ratio of these metabolites are different in the experiments recorded in Tables 2 and 3. This reflects the 250% greater protein concentration used in the former experiments in order to measure more accurately the intracellular metabolite contents. Under these conditions measurements were made after a considerable fraction of the gluconeogenic substrate had already been used, and this led to decreased output of lactate and pyruvate and a smaller [lactate]/[pyruvate] ratio.

The observed fall in oxaloacetate concentrations suggest that DCMU can cause inhibition of pyruvate carboxylase. The slight decrease in ketone-body output with increasing [DCMU] is likely to reflect a decrease in mitochondrial [acetyl-CoA] (Sugden & Williamson, 1982), and this might cause a small decrease in pyruvate carboxylase activity through its regulatory effect on the enzyme (Scrutton & Griffiths, 1981). However, this is unlikely to be sufficient to cause the observed inhibition of gluconeogenesis. Changes in mitochondrial [ATP]/[ADP] ratio are potentially able to regulate pyruvate carboxylase (Stucki *et al.*, 1972), and in the presence of alanine there is some evidence from whole-tissue measurements of ATP and ADP that this may occur (Table 2).

Role of the [NADH]/[NAD$^+$] ratio. Pyruvate carboxylase may also be regulated by the mitochondrial concentrations of malate, an inhibitor of the enzyme (Scrutton & White, 1974), and pyruvate, its substrate. The concentrations of these two metabolites are sensitive to the [NADH]/[NAD$^+$] ratio in the cytoplasm and mitochondria. The data of Fig. 5 show that DCMU causes an increase in NADH fluorescence of hepatocytes and a decrease in cytochrome *c* reduction state, as would be predicted from the site of action of the inhibitor on the respiratory chain (Halestrap, 1987). The immediacy of the effect of DCMU on both parameters demonstrates the rapid permeation of the inhibitor into the mitochondrial matrix. A rise in the cytoplasmic [NADH]/[NAD$^+$] ratio is also indicated by the increase in [lactate]/[pyruvate] ratios reported in Tables 2 and 3, whereas an increase in the mitochondrial [NADH]/[NAD$^+$] ratio is suggested by the increase in [β-hydroxybutyrate]/[acetoacetate] ratios recorded in Fig. 2. In the latter case a rise in this ratio in the presence of 0.5 mM-DCMU is only seen in the control cells. No significant effect was seen when glucagon or phenylephrine was present. However, the fluorescence measurements in Fig. 5 show that an increase in NAD(P)H does occur when glucagon or phenylephrine (results not shown) is present, and it is generally accepted that such fluorescence measurements are primarily detecting changes in the mitochondrial NADH (see Quinlan & Halestrap, 1986). Thus it would seem that the measurements of [β-hydroxybutyrate]/[acetoacetate] ratios were insufficiently precise to detect small changes in mitochondrial [NADH]/[NAD$^+$] ratio. If glucagon and phenylephrine activate the respiratory chain, as proposed, it would be expected that DCMU would cause a smaller rise in the mitochondrial [NADH]/[NAD$^+$] ratio, which might account for the difficulty in measuring any DCMU-induced increase in the [β-hydroxybutyrate]/[acetoacetate] ratio when hormones are present.

Sistare & Haynes (1985*a*,*b*) have emphasized the importance of the [NADH]/[NAD$^+$] ratio in determining the rate of gluconeogenesis from L-lactate and the effects

Table 3. Effects of DCMU on gluconeogenesis and lactate + pyruvate production by hepatocytes incubated with various substrates

Hepatocytes from 24 h-starved rats were incubated at 4 mg of protein/ml in the presence of 20 mg of defatted albumin/ml, 1 mM-sodium oleate, 0.1 μM-glucagon, the substrates indicated and, where appropriate, 0.5 mM-DCMU. The rates of gluconeogenesis were measured from the output of glucose between 30 min and 45 min, and lactate and pyruvate were measured after 45 min incubation only. Further details are given in the Experimental section. Values are given as means ± S.E.M. for seven separate experiments. The statistical significance of the effects of DCMU was measured by paired Student's t test: ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

Substrate	Rate of gluconeogenesis (nmol of glucose/min per mg of protein)		Lactate + pyruvate production (nmol/mg of protein after 45 min)		Lactate/pyruvate ratio at 45 min	
	Control	Decrease caused by 0.5 mM-DCMU	Control	Increase caused by 0.5 mM-DCMU	Control	Increase caused by 0.5 mM-DCMU
10 mM-Lactate + 1 mM-pyruvate	7.67 ± 0.97	2.38 ± 0.52**	–	–	–	–
10 mM-Alanine	4.35 ± 0.48	1.72 ± 0.24***	59.0 ± 8.6	12.0 ± 4.32*	2.59 ± 0.39	0.66 ± 0.20*
10 mM-Glycerol	2.71 ± 0.22	0.46 ± 0.11**	32.0 ± 2.8	12.2 ± 2.57**	5.16 ± 0.92	0.95 ± 0.32*
2 mM-Glutamine + 1 mM-NH_4Cl	2.08 ± 0.26	0.75 ± 0.15**	20.7 ± 4.1	4.6 ± 1.94	1.39 ± 0.38	0.67 ± 0.09***
10 mM-Fructose	15.4 ± 1.73	3.04 ± 0.83*	52.1 ± 513	55.4 ± 9.90**	6.26 ± 0.52	1.75 ± 0.65*
10 mM-Dihydroxyacetone	10.41 ± 1.06	3.48 ± 0.54**	144.5 ± 14.9	76.5 ± 6.35***	10.45 ± 1.13	4.00 ± 0.90**

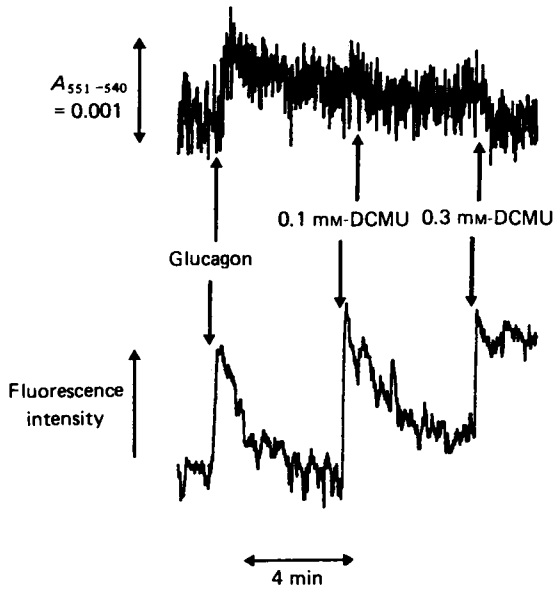

Fig. 5. Effects of DCMU on the NAD(P)H fluorescence and cytochrome *c* reduction state of isolated hepatocytes

The experimental technique was the same as that used by Quinlan & Halestrap (1986). Hepatocytes were incubated at 10 mg of protein/ml with constant stirring in the presence of 10 mM-lactate, 1 mM-pyruvate, 20 mg of defatted bovine serum albumin/ml and 1 mM-sodium oleate. Where indicated, DCMU (at the concentration shown) and 0.1 μM-glucagon were added. The experiment shown is typical of three such experiments.

of hormones on this process. They report that maximal rates of gluconeogenesis are obtained at a cytoplasmic [NADH]/[NAD^+] ratio represented by a [lactate]/[pyruvate] ratio of 10. Rates decline when the cytoplasm is either more reduced or more oxidized than this. Agius *et al.* (1986) have also suggested that the mitochondrial redox state may play an important role in the regulation of gluconeogenesis and ketogenesis. In our experiments with lactate + pyruvate as substrate, the rise in cytoplasmic [NADH]/[NAD^+] would cause inhibition, according to the data of Sistare & Haynes (1985*a*). Enhanced mitochondrial [NADH]/[NAD^+] might inhibit gluconeogenesis by increasing the malate concentration in the mitochondria and so inhibiting pyruvate carboxylase (Scrutton & White, 1974). The data of Table 2 show a small but not statistically significant increase in whole-cell malate concentrations in the presence of DCMU, which is unlikely to reflect a change in mitochondrial [malate] sufficient to cause significant inhibition of pyruvate carboxylase. Some inhibition of gluconeogenesis by 0.5 mM-DCMU was observed when 2 mM-glutamine was the substrate (Table 3). Although synthesis of glucose from this substrate does not require pyruvate carboxylase, its conversion into malate in the mitochondria requires the operation of glutamate dehydrogenase and 2-oxoglutarate dehydrogenase, and the latter in particular is sensitive to inhibition by [NADH]/[NAD^+] ratio (Denton & McCormack, 1985; Hansford, 1985). At lower concentrations of lactate or with alanine as substrate, the increase in [NADH]/[NAD^+] ratio might lower the cytoplasmic pyruvate concentration sufficiently to inhibit the transport of pyruvate into the mitochondria and its subsequent carboxylation. The flux control coefficients of these two steps are greater at lower [pyruvate] (Groen *et al.*, 1986).

Role of pyruvate kinase. If changes in [NADH]/[NAD^+] and inhibition of pyruvate carboxylase are the primary means by which the respiratory chain and gluconeogenesis interact, it might be expected that DCMU should have no effect on the rate of glucose synthesis from substrates not requiring metabolism within the mitochondria. We have studied the effects of 0.5 mM-DCMU on gluconeogenesis and lactate + pyru-

Table 4. Effects of TDGA and pent-4-enoate on the stimulation of gluconeogenesis by hormones in the presence and absence of oleate

Hepatocytes were incubated at 4 mg of protein/ml of medium containing 10 mM-L-lactate, 1 mM-pyruvate, 20 mg of defatted albumin/ml and, where indicated, 1 mM-sodium oleate, 10 μM-TDGA and 1 mM-pent-4-enoate. After incubation for 25 min, hormones were added as shown, and glucose output between 30 min and 45 min was measured. The ATP content of the 45 min $HClO_4$ extract was measured by using luciferase. Values are given as means ± S.E.M. for the numbers of observations shown in parentheses. Statistical significance of the effects of hormones (*) and inhibitors (†) were calculated by paired Student's t test: *** and ††† $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$.

	Rate of gluconeogenesis or change in rate caused by hormone (nmol/min per mg of protein)			ATP content of cells (nmol/mg of protein)		
Hormone added	Control	+10 μM-TDGA	+1 mM-Pent-4-enoate	Control	+10 μM-TDGA	+1 mM-Pent-4-enoate
Zero oleate						
None	3.96±0.22 (19)	2.46±0.07 (14)†††	2.80±0.28 (9)†††	7.10±0.34 (7)	7.623±0.38 (7)	7.11±0.63 (4)
0.1 μM-Glucagon	1.63±0.15 (19)***	0.95±0.11 (14)***†††	0.36±0.06 (9)***†††	8.01±0.43 (7)	8.25±0.34 (7)	7.14±0.50 (4)
20 μM-Phenylephrine	1.05±0.10 (17)***	0.77±0.10 (12)***	0.09±0.08 (9)†††	8.03±0.31 (6)	7.81±0.43 (6)	6.75±0.56 (4)
25 nM-Vasopressin	0.77±0.26 (9)*	0.66±0.14 (9)**	–	7.75±0.35 (2)	7.52±0.58 (2)	–
1 nM-Valinomycin	0.57±0.18 (13)**	1.19±0.15 (10)***†††	0.09±0.08 (7)†††	6.89±0.39 (6)	7.12±0.40 (6)	5.77±0.42 (4)
1 mM-Oleate						
None	7.00±0.31 (20)	2.12±0.12 (14)†††	3.25±0.30 (10)†††	7.95±0.42 (7)	7.80±0.34 (7)	7.40±0.38 (4)
0.1 μM-Glucagon	1.21±0.18 (20)***	0.95±0.13 (14)***	0.32±0.10 (10)**†††	7.90±0.45 (7)	7.55±0.33 (7)	7.63±0.60 (4)
20 μM-Phenylephrine	1.29±0.22 (18)***	0.68±0.15 (12)***†††	0.41±0.30 (10)†††	7.78±0.48 (6)	7.76±0.29 (6)	7.11±0.48 (4)
25 nM-Vasopressin	1.24±0.37 (9)*	0.71±0.28 (9)*	–	6.6±0.05 (2)	7.49±0.81 (2)	–
1 nM-Valinomycin	0.50±0.19 (11)*	1.16±0.18 (10)***†††	0.78±0.18 (5)**	6.94±0.26 (6)	6.79±0.36 (6)	7.21±0.44 (4)

vate output from glycerol, dihydroxyacetone and fructose, and the results are included in Tables 2 and 3. Gluconeogenesis from all these substrates was inhibited by 0.5 mM-DCMU, and concomitant with this there was an increase in the output of lactate + pyruvate. This is most likely to be explained by an activation of pyruvate kinase, leading to a recycling of phosphoenolpyruvate back to pyruvate. The mechanism by which pyruvate kinase is activated under these conditions is unclear, but a small but significant fall in [ATP] and rise in [ADP] was detected when dihydroxyacetone was substrate (Table 2). This could be responsible for increasing the flux through pyruvate kinase, either through a direct effect on the enzyme or indirectly through an inhibition of fructose-1,6-bisphosphatase and activation of phosphofructokinase, leading to an increase in [phosphoenolpyruvate]. No significant decrease in tissue ATP was apparent when lactate + pyruvate were the gluconeogenic substrates, which argues against pyruvate kinase activation being responsible for the inhibition of gluconeogenesis under these conditions. The slight decrease in the [ATP]/[ADP] ratio induced by 0.5 mM-DCMU when alanine was the gluconeogenic substrate (Table 2) might allow some pyruvate kinase activation. However, the small output of lactate + pyruvate relative to glucose would not suggest a major role for pyruvate kinase activation by DCMU under these conditions, either.

Role of fatty acid oxidation in the inter-relationship between the respiratory chain and gluconeogenesis. In previous papers we have presented evidence that hormonal stimulation of the respiratory chain occurs through an increase in mitochondrial matrix volume and that this is important in the stimulation of gluconeogenesis (Quinlan *et al.*, 1983; Quinlan & Halestrap, 1986). In particular, the oxidation of fatty acids is activated by small changes in the matrix volume such as those produced by gluconeogenic hormones (Halestrap & Dunlop, 1986). Thus it would be predicted that, if the stimulation of respiration is important for the stimulation of gluconeogenesis, an inhibitor of fatty acid oxidation should prevent hormonal stimulation of gluconeogenesis. Limited studies with pent-4-enoate confirmed this prediction (Quinlan & Halestrap, 1986), and in Table 4 we extend these observations and include experiments with TDGA, a potent inhibitor of carnitine acyltransferase 1 and hence of fatty acid oxidation (Tutwiler & Dellevigne, 1979; Tutwiler *et al.*, 1981; Sherratt, 1981). Neither inhibitor had a significant effect on tissue ATP content, but both greatly decreased the rate of gluconeoegenesis from L-lactate, TDGA slightly more than pent-4-enoate. This can be explained by a decrease in mitochondrial acetyl-CoA concentrations and thus decreased pyruvate carboxylase activity (Scrutton & Griffiths, 1981). In the presence of pent-4-enoate the stimulation of gluconeogenesis by glucagon or phenylephrine is largely abolished in either the presence or the absence of oleate. Stimulation by valinomycin is abolished in the absence of oleate, but slightly increased in its presence. In the presence of 10 μM-TDGA, sufficient to give maximal inhibition of fatty acid oxidation and gluconeogenesis (Tutwiler & Dellevigne, 1979; Tutwiler *et al.*, 1981), hormonal stimulation of gluconeogenesis is still apparent in either the presence or the absence of oleate, and the effects of valinomycin are greater in both cases. One explanation for the ability of hormones to stimulate

gluconeogenesis in the presence of TDGA, but to have a very small effect in the presence of pent-4-enoate, could be as follows. Pent-4-enoate inhibits the β-oxidation pathway almost totally and would therefore prevent any hormonal stimulation of fatty acid oxidation. Any residual effect could be due to the stimulation of the oxidation of other substrates through a volume-mediated effect on electron flow through the respiratory chain (Halestrap, 1982; Armston *et al.*, 1982; Quinlan & Halestrap, 1986). However, when TDGA is present some carnitine-independent fatty acid oxidation can occur (Tutwiler *et al.*, 1981; Schudt & Simon, 1984; Wolf & Engel, 1985), and it would be expected that this will still be sensitive to volume-mediated hormonal (or valinomycin) stimulation. Why valinomycin should stimulate more in the presence of TDGA is puzzling, but may reflect the ability of valinomycin to oxidize the mitochondrial NADH (Quinlan & Halestrap, 1986).

General discussion

We have provided evidence in the past that, through a co-ordinated stimulation of fatty acid oxidation, respiration and pyruvate carboxylation, hormones can stimulate gluconeogenesis without major changes in [ATP]/[ADP] or [NADH]/[NAD^+] ratios (Quinlan & Halestrap, 1986; Halestrap & Dunlop, 1986). The present paper confirms the existence of such a link between the respiratory chain and gluconeogenesis, and from the calculated flux control coefficients suggests that it is a site of considerable regulatory potential. It is of interest that several well-known hypoglycaemic agents are mild respiratory-chain inhibitors (Sherratt, 1981), and the data in the present paper may give some insight into how they work.

This work was supported by grants from the Medical Research Council and the British Diabetic Association, and a research studentship to P.T.Q. from the Science and Engineering Research Council. We thank Professor R. M. Denton for advice and encouragement.

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Received 16 March 1987/11 June 1987; accepted 16 July 1987