Effect of dexamethasone on gluconeogenesis, pyruvate kinase, pyruvate carboxylase and pyruvate dehydrogenase flux in isolated hepatocytes

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Treatment of 18 h-starved rats with dexamethasone and subsequent isolation and incubation of the hepatocytes in the presence of the steroid increased gluconeogenic flux with both 1.0 mM pyruvate and 1.0 mM lactate plus 0.2 mM pyruvate as the substrate. The magnitude of stimulation was comparable with both substrates. The increase in glucose output was accompanied by an increased flux through pyruvate carboxylase, although the absolute flux and magnitude were considerably less in the presence of the more reduced substrate. The effect of the steroid on the flux through pyruvate dehydrogenase was substrate-dependent, an inhibition occurring with the more oxidized substrate. There was no effect of steroid treatment on

[1-¹⁴C]lactate or pyruvate oxidation or on tricarboxylic-acid-cycle flux as measured by [3-¹⁴C]pyruvate oxidation. Dexamethasone treatment resulted in a parallel increase in both pyruvate kinase flux and glucose synthesis with both substrates employed, indicating that the steroid had no effect on the partitioning of phosphoenolpyruvate between pyruvate and lactate formation and gluconeogenesis. Similarly there was no effect of the steroid on either the activity ratio or the total pyruvate kinase activity in the cells. It is suggested that the acute effect of the dexamethasone to increase gluconeogenesis resides at the level of phosphoenolpyruvate formation, i.e. pyruvate carboxylase and possibly phosphoenolpyruvate carboxykinase.

INTRODUCTION

It is well established that glucocorticoids have both a direct effect and permissive role in the stimulation of gluconeogenesis, the latter resulting from a modulation of the sensitivity of the liver to glucagon and catecholamines [1–8]. Numerous studies using both isolated hepatocytes and perfused livers have pointed to the reactions involved in the first futile cycle of the pathway between pyruvate and phosphoenolpyruvate (PEP) as being the major site of control [3,5–7]. From control-strength-analysis studies it has been suggested that the site of control is distributed between pyruvate kinase and the reactions involved in PEP formation, i.e. pyruvate carboxylase and PEPCK [9]. The mechanisms underlying these direct effects remain poorly understood. Attempts to measure changes in the activity of pyruvate kinase have been ambiguous. Johnson et al. [10] demonstrated that perfusion of rabbit liver with dexamethasone or injection of the steroid into the intact animal resulted in a rapid decrease in the $V_{\max}$ of the enzyme measured in the presence of the allosteric activator fructose 1,6-bisphosphate, although no change was apparent in the total enzyme protein. In contrast, Blair and Kletzien [11] were unable to show any change in total activity of the enzyme in cultured rat hepatocytes incubated with dexamethasone, although a 30% increase in the $K_{0.5}$ for PEP was detected; however, in the same study, steroid replacement therapy in adrenalectomized rats or treatment of freshly isolated hepatocytes with dexamethasone failed to have any effect on pyruvate kinase activity. Similarly, although it is widely accepted that the induction of PEPCK by glucocorticoids is part of the mechanism by which PEP formation and glucose synthesis can be stimulated chronically [12–15], a number of reports suggest that this is not responsible for the earliest acute effects of glucocorticoids on gluconeogenesis [9,14]. Alterations in pyruvate carboxylase activity measured in intact mitochondria isolated from rats treated with steroid hormones correlate well with the known short-term effects of glucocorticoids on glucose synthesis, in terms of both

the selectivity and sensitivity to steroids and the time of onset of the response [16–20]. This stimulation has been attributed to the combined effect of a number of factors, including an increase in the intramitochondrial concentrations of acetyl-CoA, Mg^{2+} and ATP and an increased ATP/ADP ratio, a decrease in the matrix glutamate concentration and an elevated rate of transport across the mitochondrial inner membrane [7,16–20]. However, the relevance of these studies to the stimulation of gluconeogenesis remains unclear, as the possibility exists that some of these effects may be artifacts induced by the mitochondrial preparation procedure rather than a true activation *in vivo* [21–23]. Therefore the aim of this study was severalfold: first, to investigate the effect of dexamethasone treatment on the kinetics of pyruvate kinase and to correlate this with the flux through the enzyme in intact cells, to determine the possible contribution made by this enzyme to the stimulation of gluconeogenesis; second, to examine the effects of the steroid on the flux through pyruvate carboxylase *in situ* and to compare any changes with conclusions drawn from studies with isolated mitochondria and control strength analysis; finally, to measure the flux through pyruvate dehydrogenase (PDH) to determine whether an inhibition of activity might result in a sparing effect with an increased supply of substrate for gluconeogenesis.

EXPERIMENTAL

Materials

Dexamethasone acetate, glucagon, dichloroacetate, collagenase, $NaH^{14}CO_3$ and [U-¹⁴C]glucose were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). [1-¹⁴C]Pyruvate and [3-¹⁴C]pyruvate were from NEN Du Pont (Stevenage, Herts., U.K.). [1-¹⁴C]Lactate was from ICN Biomedicals (High Wycombe, Bucks., U.K.). [1-¹⁴C]Alanine was from Amersham International (Amersham, Bucks., U.K.). Dexamethasone sodium phosphate was a gift from Merck, Sharp and Dohme (Hoddesdon, Herts., U.K.). All other chemicals were of AnalaR

Figure 1 Distribution of label from $\text{NaH}^{14}\text{CO}_3$ (a) and $[1-^{14}\text{C}]$ pyruvate and $[3-^{14}\text{C}]$ pyruvate (b)

Both panels assume randomization of the label in C-1 and C-4 of oxaloacetate as a result of equilibrium with fumarate. In (b) incorporation of label from $[1-^{14}\text{C}]$ pyruvate is shown as ^{14}C , whereas incorporation of label from $[3-^{14}\text{C}]$ pyruvate is shown as ^{14}C where it enters via acetyl-CoA and ^{14}C where it enters via oxaloacetate. Abbreviations: PC, pyruvate carboxylase; ME, malic enzyme; PK, pyruvate kinase; MDH, malate dehydrogenase.

grade or similar purity from either BDH Chemicals (Enfield, Middx., U.K.) or Sigma.

Preparation and incubation of hepatocytes

Male Sprague-Dawley rats (180–220 g body wt.) were starved for 18 h and injected with dexamethasone acetate (15 $\mu\text{mol}/100$ g body wt.) or 0.9% NaCl. After a 90 min treatment with the steroid, hepatocytes were prepared as described previously [7]. Preliminary studies attempting to set up a complete system *in vitro* to measure the effects of steroids on gluconeogenesis in freshly isolated hepatocytes produced variable and inconsistent effects. Therefore the short treatment regimen *in vivo* employed previously [7] was used. Dexamethasone sodium phosphate (1 μM final concn.) was included in all preparation and incubation media in contact with cells prepared from steroid-treated animals to ensure the maintenance of the response. The hepatocytes were kept on ice before the start of the incubations. Except for the measurements of pyruvate kinase activity, the cells were suspended to a final concentration of 12 mg wet wt./ml in a modified Krebs/Henseleit buffer containing 25 mM- $\text{NaH}^{14}\text{CO}_3$, 20 mM-Hepes, pH 7.4 and 1.5% defatted BSA and gassed with 100% oxygen. Where indicated, dichloroacetate

was present from the start of the incubations at a final concentration of 2 mM. Triplicate incubations were carried out in 25 ml Erlenmeyer flasks for 15 min, with the order of the incubations randomized with respect to the labels and cell preparations.

Pyruvate kinase measurements

The cells were incubated in Krebs-Henseleit bicarbonate buffer (40 mg wet wt./ml) in the presence of 20 mM-lactate/4 mM-pyruvate under an atmosphere of O_2/CO_2 (19:1). After 30 min, glucagon (final concn. 0.1 μM) or vehicle was added. Samples were removed 3 min later, added to 3 ml of buffer containing 100 mM-NaF, 10 mM-EDTA and 10 mM-Tes, pH 7.5, and immediately frozen in liquid N_2 . The pyruvate kinase was partially purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation and assayed as described previously [24].

Pyruvate kinase flux measurements

Pyruvate kinase flux (J_{PK}) was measured by the incorporation of $\text{NaH}^{14}\text{CO}_3$ into glucose, pyruvate and lactate as initially described by Rognstad [25]. $^{14}\text{CO}_2$ is incorporated into oxaloacetate into C-4 by pyruvate carboxylase; however, the label is randomized into C-1 and C-4 by equilibration with fumarate (Figure 1a). During the PEPCK reaction the label is lost from C-4 as $^{14}\text{CO}_2$, leaving $[1-^{14}\text{C}]$ PEP, which can then be either converted into $[^{14}\text{C}]$ glucose or $[1-^{14}\text{C}]$ pyruvate and -lactate. As shown in Figure 1(a), malic enzyme could also potentially contribute to the pool of labelled pyruvate and lactate formed and therefore lead to an overestimate of pyruvate kinase flux. However, there is strong evidence to suggest that the contribution from malic enzyme is negligible. First measurements of the activity of malic enzyme in rat hepatocytes reveal that the activity is very low in the fasted state [26]. Furthermore, in experiments where 2,4-dihydroxybutyrate, an inhibitor of malic enzyme, has been employed, it has been found to have no effect on pyruvate kinase flux with 20 mM-pyruvate as the substrate [27]. Similarly, the inhibitor had no effect on the partitioning of PEP between gluconeogenesis and pyruvate in the hyperthyroid animal, a condition in which malic enzyme is induced and might be expected to be active [28,29].

Incubations were started by addition of 1.25 ml of hepatocytes (12 mg wet wt./ml) to an equal volume of modified Krebs/Henseleit buffer containing 20 mM-Hepes, pH 7.4, 1.5% defatted BSA, 25 mM- $\text{NaH}^{14}\text{CO}_3$ (sp. radioactivity 0.15 $\mu\text{Ci}/\mu\text{mol}$) and either 2.0 mM-lactate plus 0.4 mM-pyruvate or 2.0 mM-pyruvate as substrates. The cells were maintained under an atmosphere of 100% oxygen to prevent isotopic dilution of the $\text{NaH}^{14}\text{CO}_3$ in the medium with unlabelled CO_2 in the gas phase. At the final cell concentration employed, less than 20% of the total substrate added was used. The incubations were stopped by addition of 10% 2.7 M HClO_4 and then bubbled with CO_2 to drive off unincorporated $^{14}\text{CO}_2$. After neutralization of the samples, the incorporation of label into glucose, pyruvate and lactate was determined. Radioactive glucose was measured by its exclusion from a mixture of ion-exchange resins (Dowex 50W-X8/Amberlite IRA 68, 1:1). Portions (400 μl) of the neutralized deproteinized extracts were incubated for 1 h with 120 μl of a conversion mixture containing 1.5 mM- $(\text{NH}_4)_2\text{SO}_4$, 10 mM- MgCl_2 , 10 mM-ATP, 10 mM-Tris/HCl, pH 7.0 and 5.0 mM-glucose in the absence and presence of hexokinase (0.154 unit/ml). Hexokinase was added to convert glucose into glucose 6-phosphate, to distinguish between radioactive glucose and other non-ionic radioactive compounds. A 400 μl portion of the

mixture was then added to 0.4 g of the ion-exchange-resin mixture together with 500 μ l of water. After shaking for 1 h, samples of the supernatant were counted for radioactivity in a Beckman LS 2800 scintillation counter, and the glucose was determined by the difference in radioactivity in the samples incubated with and without hexokinase. [14 C]Glucose standards were included to correct for the conversion and recovery of glucose. Glucose conversions and recoveries were 91.4 ± 1.3 and $93.1 \pm 1.9\%$ ($n = 24$) respectively. From the enzymic measurement of glucose accumulated in the medium over the 15 min incubation period, the specific radioactivity of the glucose was calculated and the specific radioactivity of the radiolabelled PEP precursor was determined as 50% of the glucose specific radioactivity [25]. The flux of label through to glucose (J_{glucose}) is expressed in terms of nmol of PEP converted/15 min per mg cell wet wt.

Because a lower concentration of cells was used over a shorter incubation period than in the original method, it was found that the labelled lactate and pyruvate formed could not be determined by ion-exchange chromatography [25], and therefore a more specific enzymic method was employed. Incorporation of $\text{NaH}^{14}\text{CO}_3$ into [14 C]-pyruvate and -lactate was measured by the release of $^{14}\text{CO}_2$ from neutralized cell extracts in the presence of lactate oxidase and pyruvate oxidase. Portions (300 μ l) of the cell extracts were incubated with 300 μ l of reaction mixture (160 mM- KH_2PO_4 , pH 8.4, 1.57 mM- MnCl_2 , 0.02 mM-FAD, 0.1 mM-pyridoxal 5-phosphate and 4.7 units of catalase/ml) at 37 °C in glass vials fitted with Subaseal rubber stoppers and a centre well containing 250 μ l of Hyamine 10X (diluted 1:1 with methanol). The incubations were carried out both in the absence of added enzymes and in the presence of pyruvate oxidase and a mixture of pyruvate oxidase and lactate oxidase (final concn. of both enzymes 0.3 unit/ml) to determine background $^{14}\text{CO}_2$ release and specific release from [14 C]pyruvate and [14 C]-pyruvate plus [14 C]lactate. After 4 h the reaction was stopped by injection of 150 μ l of 2.7 M HClO_4 and 120 mM NaHCO_3 was injected to drive off $^{14}\text{CO}_2$ still in solution. The $^{14}\text{CO}_2$ was trapped over 90 min and the radioactivity in the Hyamine determined. [14 C]Pyruvate and [14 C]lactate plus pyruvate standards were included in the assay to determine the efficiency of recovery of the label. These were $72.3 \pm 1.4\%$ and $77.0 \pm 1.4\%$ ($n = 24$) for pyruvate and lactate respectively and were allowed for in all calculations. To determine the efficiency of the trapping of [14 C]-pyruvate and -lactate in the pool and to estimate the extent of re-cycling of the label, the cells were incubated with a trace amount of [14 C]alanine (0.2 $\mu\text{Ci/ml}$; sp. radioactivity 51 $\mu\text{Ci}/\mu\text{mol}$) to label the intracellular lactate and pyruvate pool [25]. After 5 min the reaction was terminated and $^{14}\text{CO}_2$, [14 C]glucose and [14 C]-lactate and -pyruvate production were determined as described above. The percentage reutilization was calculated from an alanine partitioning factor, calculated as the ratio of the production of labelled [CO_2 + glucose + lactate + pyruvate]/[lactate + pyruvate] as described in [25]. The alanine partitioning factor was found to be 1.08 ± 0.01 ($n = 4$) with 1.0 mM lactate/0.2 mM pyruvate as the substrate, giving a reutilization of $7.1 \pm 0.7\%$ of the label incorporated into lactate and pyruvate for four different cell preparations. The result was not significantly different with 1.0 mM pyruvate as the substrate. This value is substantially less than the 30–40% reutilization estimated by Rognstad [25] with 20 mM pyruvate alone as the substrate and a high cell density, despite the fact that the extracellular pool of lactate and pyruvate was greatly diminished in our experiments. This suggests that there may not be complete equilibration between the labelled pyruvate and lactate formed from the $\text{NaH}^{14}\text{CO}_3$ and the external unlabelled pool. These data indicate that the underestimation of pyruvate kinase flux under

these conditions is minimal, and no further corrections for incomplete trapping were employed.

It has been suggested that measuring the incorporation of labelled PEP into only pyruvate and lactate may lead to a substantial underestimation of J_{PK} as a result of equilibration of the pyruvate with alanine, in addition to lactate [29,30]. To resolve this problem, the incorporation of PEP into [14 C]-alanine was measured by converting the alanine into [14 C]-pyruvate by inclusion of alanine aminotransferase (7.8 units) and 2-oxoglutarate (15 mM final concn.) in the pyruvate assay described above. With 1 mM lactate/0.2 mM pyruvate as the substrate, the incorporation of radioactivity into alanine accounted for 7.4% ($n = 6$) of the total activity in lactate and pyruvate. With 1 mM pyruvate as the substrate, the incorporation was outside the limits of detection of the assay. Therefore, under the conditions used, it is concluded that the accumulation of label into alanine does not make a substantial contribution to J_{PK} with low substrate concentrations, and no attempts were made to correct for this. Pyruvate kinase flux (J_{PK}) is expressed in terms of nmol of PEP converted into lactate plus pyruvate/15 min per mg cell wet wt. The flux through PEPCK (J_{PEPCK}) was calculated as the sum of $J_{\text{glucose}} + J_{\text{PK}}$ [30].

PDH and pyruvate carboxylase flux measurements

The fluxes through pyruvate carboxylase (J_{PC}) and PDH (J_{PDH}) were measured essentially as described previously [31,32]. The cells were incubated in the presence of [14 C]pyruvate (sp. radioactivity 0.1 $\mu\text{Ci}/\mu\text{mol}$), [14 C]pyruvate (sp. radioactivity 0.4 $\mu\text{Ci}/\mu\text{mol}$), or a mixture of [14 C]pyruvate and [14 C]acetate (sp. radioactivities 0.2 $\mu\text{Ci}/\mu\text{mol}$) in a total volume of 1.5 ml in 25 ml Erlenmeyer flasks stoppered with Subaseal rubber stoppers. In incubations containing 1.0 mM lactate and 0.2 mM pyruvate, [14 C]lactate was unavailable to determine tricarboxylic-acid-cycle activity (J_{TCA}), and therefore corrections were made for the $^{14}\text{CO}_2$ released from [14 C]lactate by using the $^{14}\text{CO}_2$ released from [14 C]lactate multiplied by the [14 C]pyruvate/[14 C]pyruvate ratio. After 15 min, 0.25 ml of Hyamine 10X (diluted 1:1 with methanol) was injected into a centre well, followed immediately by 0.15 ml of 2.7 M- HClO_4 to terminate the reaction. $^{14}\text{CO}_2$ produced was collected over the next 90 min. $\text{NaH}^{14}\text{CO}_3$ standards were included in the incubations to determine the efficiency of trapping. Overall, for 24 experiments a $^{14}\text{CO}_2$ recovery of $83.6 \pm 2.0\%$ was found. The deproteinized extracts were stored at -20°C until assayed for [14 C]glucose production as described above.

Label derived from either [14 C]pyruvate or [14 C]lactate is lost as $^{14}\text{CO}_2$ via the action of PDH or is incorporated into [14 C]oxaloacetate via pyruvate carboxylase (Figure 1b). Assuming randomization of the label in C-1 and C-4 of oxaloacetate by equilibration with fumarate, the ^{14}C label in the oxaloacetate may then either be lost as $^{14}\text{CO}_2$ during the first turn of the tricarboxylic acid cycle or through the action of malic enzyme and PEPCK ($J_{\text{ME+PEPCK}}$), or be incorporated into glucose, lactate and pyruvate. Therefore the flux through PDH can be determined as:

$$J_{\text{PDH}} = \text{Total } ^{14}\text{CO}_2 \text{ from } ^{14}\text{C-labelled} \\ \text{substrates} - (J_{\text{TCA}} + J_{\text{PEPCK+ME}})$$

where J_{TCA} was estimated by the release of $^{14}\text{CO}_2$ from [14 C]-pyruvate, and $J_{\text{PEPCK+ME}}$ was calculated from $J_{\text{glucose}} \times F$ [31,32]. The factor F was derived from the incorporation of label into [pyruvate + lactate + glucose]/[glucose] and allows for the amount of [14 C]oxaloacetate formed which is recycled into pyruvate and lactate via pyruvate kinase rather than being

converted into glucose [32]. Although the branched-chain 2-oxo acid dehydrogenase complex in liver can utilize pyruvate as the substrate and therefore may lead to an overestimate of the PDH flux, with the low levels of pyruvate and lactate used in the present study it is unlikely to represent an error of more than a few per cent [33], and therefore no attempt has been made to correct for this.

Pyruvate carboxylase flux was determined as:

$$J_{PC} = \text{total pyruvate metabolized} - J_{PDH} \quad [32].$$

The total pyruvate metabolized was calculated essentially as described previously [32], except that an additional factor (P) was added to account for the pyruvate which was recycled via pyruvate kinase. This was calculated from the product of the total $[1-^{14}\text{C}]$ pyruvate or $[1-^{14}\text{C}]$ lactate incorporated into glucose and the ratio $[\text{pyruvate} + \text{lactate}]/[\text{glucose}]$, as determined in the pyruvate kinase flux experiments.

Total pyruvate metabolized = J_{glucose} + total $^{14}\text{CO}_2$ released + P . The results shown are means $\pm$ S.E.M. for six different cell preparations. Statistical analysis was performed by two-way analysis of variance.

RESULTS AND DISCUSSION

Effect of dexamethasone on pyruvate kinase activity

Figure 2 shows the effect of dexamethasone treatment of pyruvate kinase activity in $(\text{NH}_4)_2\text{SO}_4$ cell extracts. This was compared with that of 0.1 μM glucagon as a positive control. The presence of glucagon significantly decreased the pyruvate kinase activity ratio and pyruvate kinase activity at all concentrations up to and including 1.0 mM PEP in both control cells and cells prepared from the dexamethasone-treated animals. In agreement with previous studies [24,34], the $K_{0.5}$ for PEP was increased approx. 2.5-fold in the presence of glucagon in both cell types, the apparent K_m increasing from 0.51 mM to 1.28 mM and 0.55 mM to 1.28 mM in the control and dexamethasone-treated cells

Figure 2 Effect of dexamethasone and glucagon on pyruvate kinase activity

The protocol was as described in the Experimental section. Total pyruvate kinase activity measured in the presence of 5 mM PEP and 20 μM fructose 1,6-bisphosphate was 0.19 ± 0.02 and 0.17 ± 0.02 for the enzyme prepared from control and dexamethasone-treated cells respectively. The activity ratio, expressed as the ratio of the activity of 0.5 mM-PEP to the total, was 0.47 ± 0.03 and 0.19 ± 0.03 for control cells in the absence and presence of glucagon and 0.45 ± 0.03 and 0.24 ± 0.03 for dexamethasone-treated cells in the absence and presence of glucagon respectively. Results shown are means $\pm$ S.E.M. for six different cell preparations. Black symbols represent pyruvate kinase activity measured in the presence of 20 μM fructose 1,6-bisphosphate. $\bullet$, $\circ$, Control; $\blacksquare$, $\square$, 0.1 μM glucagon; $\blacktriangle$, $\triangle$, 1 μM dexamethasone; $\blacktriangledown$, $\triangledown$, 0.1 μM glucagon + 10 μM dexamethasone.

respectively in the absence and presence of glucagon. In neither cell type was there any effect of glucagon on the total activity of the enzyme measured in the presence of 20 μM fructose 1,6-bisphosphate at any concentration of PEP. In contrast with glucagon, dexamethasone treatment alone had no significant effect on the activity ratio or the pyruvate kinase activity measured at any concentration of PEP tested, in either the absence or presence of fructose 1,6-bisphosphate, suggesting there was no change in the phosphorylation state of the enzyme after steroid treatment. This agrees with the work of Blair and Kletzien [11], using freshly isolated hepatocytes from normal or adrenalectomized animals, but contrasts with their studies using cultured cells. There was a small, but insignificant (12%) change in the total assayable activity of the enzyme, which may reflect the change noted by Johnson et al. [10]. However, this is unlikely to make a major contribution to the stimulation of gluconeogenesis after steroid treatment, as it is evident that the enzyme is severely depressed in the fasted state and it is unlikely to be working under V_{max} conditions [35]. These results indicate that if the effect of dexamethasone treatment does reside at the level of pyruvate kinase [8,9,36], then it is the result of changes in the intracellular concentrations of allosteric effectors rather than alterations in the total enzyme activity or phosphorylation state.

Effect of dexamethasone on pyruvate kinase flux with 1.0 mM lactate/0.2 mM pyruvate as substrates

From the work of Rognstad and Katz [35] it is evident that, although no changes in pyruvate kinase activity could be measured in the isolated enzyme, this does not preclude a change in the flux through the enzyme in the intact cell. No previous studies have been carried out to determine directly the flux through pyruvate kinase in intact cells in the presence of dexamethasone, and therefore a direct comparison of pyruvate kinase flux rates with control-strength-analysis studies is not possible. Therefore experiments were carried out to measure directly pyruvate kinase flux with a lactate/pyruvate mixture which was close to physiological.

Table 1 shows the effect of dexamethasone treatment of the rats on lactate and pyruvate formation from $\text{NaH}^{14}\text{CO}_3$ in the presence of 1.0 mM lactate plus 0.2 mM pyruvate. The control cells showed a considerable conversion of $[^{14}\text{C}]$ PEP into lactate and pyruvate. The J_{PK}/J_{PEPCK} ratio indicates that approx. 35%

Table 1 Effect of dexamethasone on pyruvate kinase flux with 1.0 mM lactate and 0.2 mM pyruvate as the substrate

Hepatocytes were prepared as described in the Experimental section and incubated with $\text{NaH}^{14}\text{CO}_3$ plus 1.0 mM lactate/0.2 mM pyruvate as the substrate in the presence of 10 μM dexamethasone. After 15 min the reaction was terminated and the incorporation of $^{14}\text{CO}_2$ into pyruvate, lactate and glucose was determined. J_{PK} , J_{glucose} and J_{PEPCK} were measured as described in the Experimental section. Results are expressed as nmol of $[^{14}\text{C}]$ PEP incorporated/15 min per mg wet wt. of cells and are means $\pm$ S.E.M. for six different cell preparations: * $P < 0.05$; ** $P < 0.01$.

Treatment ...	Control	Dexamethasone
Pyruvate formed	2.2 ± 0.8	$4.1 \pm 1.1^{**}$
Lactate formed	2.6 ± 0.4	4.6 ± 0.7
J_{PK}	4.8 ± 1.2	$8.1 \pm 1.2^{**}$
J_{glucose}	9.1 ± 1.5	$14.2 \pm 1.3^*$
J_{PEPCK}	13.9 ± 1.5	$22.3 \pm 2.0^{**}$
J_{PK}/J_{PEPCK} (%)	34.1 ± 7.1	35.0 ± 4.8
$J_{\text{glucose}}/J_{PEPCK}$ (%)	65.9 ± 7.1	65.0 ± 4.9

of the total [^{14}C]PEP formed is converted into [$1\text{-}^{14}\text{C}$]-pyruvate plus lactate, whereas 65% of the PEP is converted into glucose ($J_{\text{glucose}}/J_{\text{PEPCK}}$). This value is close to that reported by Haynes and Picking [30], using a similar method, and also to the 41% obtained by using ^{13}C n.m.r. for livers perfused with either alanine or pyruvate as the substrates in the presence of both ethanol and NH_4Cl [37]. It is midway between the 10% pyruvate kinase flux estimated in the presence of 20 mM lactate and the 50–60% measured in the presence of 20 mM pyruvate [25,27,35]. In contrast with the conclusions drawn from control strength analysis studies by Sistare and Haynes [9], direct measurement of pyruvate kinase flux indicated that dexamethasone treatment resulted in a significant increase in J_{PK} (69%) rather than inhibiting flux through the enzyme. This was accompanied by a comparable significant increase in J_{glucose} (56%). The ratios of $J_{\text{PK}}/J_{\text{PEPCK}}$ and $J_{\text{glucose}}/J_{\text{PEPCK}}$ were not significantly changed by the presence of dexamethasone, indicating that no change in the partitioning of PEP between lactate plus pyruvate and glucose occurred. This confirms that dexamethasone has no significant effect on the kinetics of pyruvate kinase and suggests that the enzyme is controlled predominantly by the availability of its substrate, PEP, under these conditions. In this respect an increase in PEP has previously been demonstrated after steroid treatment of rats [3,6,7]. Therefore it can be concluded that the major effect of dexamethasone to stimulate glucose synthesis (and pyruvate plus lactate formation) at physiological substrate concentrations is the result of the increase in J_{PEPCK} (Table 1).

Effect of dexamethasone on pyruvate kinase flux with 1.0 mM pyruvate as the substrate

Sistare and Haynes [8,9,36] have demonstrated that the effect of dexamethasone on gluconeogenesis is most apparent with either low pyruvate as a substrate or when there is a high lactate/pyruvate ratio. The use of pyruvate alone as the substrate has also been shown to increase the flux through pyruvate kinase in intact cells [25,35]. Therefore we measured the flux through pyruvate kinase with 1 mM pyruvate as the substrate to determine whether an inhibition of flux could contribute to the stimulation of glucose synthesis by dexamethasone under more oxidized conditions. The results are shown in Table 2. In control cells the addition of the more oxidized substrate substantially increased pyruvate kinase flux as compared with cells incubated in the presence of 1.0 mM lactate/0.2 mM pyruvate. The percentage of total PEP formed which was converted into lactate plus pyruvate ($J_{\text{PK}}/J_{\text{PEPCK}}$) was increased from $34 \pm 7\%$ to $53 \pm 3\%$ ($P < 0.001$,

$n = 12$), such that there was an equal partitioning of the PEP between glucose ($J_{\text{glucose}}/J_{\text{PEPCK}}$) and pyruvate plus lactate formation when 1.0 mM pyruvate was employed as the substrate.

This agrees with previous observations and supports the concept of control of pyruvate kinase flux by the oxidation–reduction state of the cytoplasm [27]. Treatment of the rat with dexamethasone produced a comparable increase in gluconeogenesis as when a mixture of lactate plus pyruvate was employed as the substrate (65% stimulation, compared with 56%). As with the more reduced substrate, dexamethasone also significantly increased J_{PK} , the mean increase being 40%. The $J_{\text{PK}}/J_{\text{PEPCK}}$ and $J_{\text{glucose}}/J_{\text{PEPCK}}$ ratios indicate that there was no significant difference in the partitioning of label between lactate plus pyruvate and glucose in control and dexamethasone-treated hepatocytes, confirming that the steroid also has no significant effect on pyruvate kinase activity with the more oxidized substrate and that the increased flux reflects an increased J_{PEPCK} .

Effect of dexamethasone on glucose synthesis from a mixture of [$1\text{-}^{14}\text{C}$]lactate plus pyruvate and from [$1\text{-}^{14}\text{C}$]pyruvate alone

To determine whether the increase in J_{PEPCK} was the result of an increased substrate supply, or an effect on dexamethasone on the enzyme itself, the flux through pyruvate carboxylase was measured. Table 3 compares the effect of dexamethasone with 1.0 mM lactate plus 0.2 mM pyruvate and with 1.0 mM pyruvate as substrates. The effect of the hormone was compared with that of 2.0 mM dichloroacetate as a positive control, as this is known both to increase the activity of the active form of PDH (PDH_a) as a result of an inhibition of PDH kinase [38] and to increase the flux through the enzyme *in situ* [31]. Treatment of the rat with dexamethasone significantly increased glucose formation from both substrates, the mean percentage stimulation being comparable (39% and 42% with pyruvate and with lactate plus pyruvate respectively). When dichloroacetate was added to the cells, no significant change in glucose output occurred with 1.0 mM pyruvate as the substrate; however, glucose synthesis from the more reduced substrate was inhibited by 18%. The latter is consistent with the inhibition of glucose synthesis noted by Crabb et al. [39] in cells incubated with normal bicarbonate concentrations and has been ascribed to an inhibition of pyruvate carboxylation as a result of the conversion of dichloroacetate into glyoxalate and oxalate.

Total $^{14}\text{CO}_2$ released from [$1\text{-}^{14}\text{C}$]lactate plus pyruvate and [$1\text{-}^{14}\text{C}$]pyruvate alone

In control cells incubated with a mixture of lactate plus pyruvate there was a significant inhibition of $^{14}\text{CO}_2$ production compared with the oxidation of pyruvate alone, the respective values for the combined controls being 15.5 ± 0.6 and 20.1 ± 0.8 ($P < 0.01$, $n = 12$) nmol/15 min per mg wet wt. A similar trend has been observed previously in both perfused livers [40] and hepatocytes [31] and is likely to be due to a number of factors, including a decreased effective pyruvate concentration for mitochondrial pyruvate oxidation and also a shift in the cytosolic malate/oxaloacetate equilibrium in favour of malate production, with a consequent decreased flux through PEPCK. Treatment of the cells with an optimal concentration of dichloroacetate to increase PDH activity [31] significantly increased $^{14}\text{CO}_2$ production from [$1\text{-}^{14}\text{C}$]pyruvate (48%), but there was no effect on the oxidation of [$1\text{-}^{14}\text{C}$]lactate plus pyruvate, although an increase in PDH_a is known to occur with reduced substrates [41]. Although dexamethasone has been demonstrated to increase [$1\text{-}^{14}\text{C}$]pyruvate decarboxylation in isolated mitochondria [16–19], it had no significant effect on cellular $^{14}\text{CO}_2$ output with either substrate.

Table 2 Effect of dexamethasone on pyruvate kinase flux with 1.0 mM pyruvate as the substrate

The protocol was as described in Table 1, except that 1.0 mM pyruvate was used as the substrate. Results are expressed as nmol of [^{14}C]PEP incorporated/15 min per mg wet wt. of cells and are means $\pm$ S.E.M. for six different cell preparations: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Treatment ...	Control	Dexamethasone
Pyruvate formed	8.1 ± 1.2	$10.3 \pm 1.4^{**}$
Lactate formed	3.4 ± 0.9	5.7 ± 1.5
J_{PK}	11.5 ± 1.7	$16.0 \pm 2.3^*$
J_{glucose}	9.6 ± 0.8	$15.9 \pm 1.4^{**}$
J_{PEPCK}	21.0 ± 2.1	$31.9 \pm 2.9^{***}$
$J_{\text{PK}}/J_{\text{PEPCK}}$ (%)	53.4 ± 3.4	49.3 ± 4.0
$J_{\text{glucose}}/J_{\text{PEPCK}}$ (%)	49.3 ± 4.0	50.7 ± 4.0

Table 3 Effect of dexamethasone and dichloroacetate on pyruvate metabolism

Hepatocytes were incubated for 15 min with the appropriate additions as described in the Experimental section. Dexamethasone (Dex) or dichloroacetate (DCA) was added to final concentrations of 1 μ M or 2 mM respectively. The fluxes through pyruvate kinase and malic enzyme ($J_{\text{PEPCK}} + J_{\text{ME}}$), pyruvate dehydrogenase (J_{PDH}) and pyruvate carboxylase (J_{PC}) were calculated as described in the Experimental section. Results are expressed as means $\pm$ S.E.M. for six different cell preparations: * $P < 0.05$; ** $P < 0.01$.

Substrate	Treatment	Metabolism (nmol/15 min per mg wet wt.)				<i>F</i> factor	$J_{\text{PK}} + J_{\text{ME}}$	J_{PDH}	J_{PC}
		[1- 14 C]Pyruvate incorporation into glucose	[1- 14 C]Pyruvate oxidized	[3- 14 C]Pyruvate oxidized	Total pyruvate metabolized				
1.0 mM pyruvate	Cont	4.3 $\pm$ 0.6	21.1 $\pm$ 1.1	6.0 $\pm$ 0.4	30.4 $\pm$ 1.8	2.20 $\pm$ 0.15	9.2 $\pm$ 0.8	5.9 $\pm$ 0.3	24.5 $\pm$ 1.8
	Dex	6.0 $\pm$ 0.6*	22.9 $\pm$ 2.0	6.1 $\pm$ 0.8	34.8 $\pm$ 3.0	2.04 $\pm$ 0.39	12.0 $\pm$ 1.0	4.8 $\pm$ 0.5*	30.1 $\pm$ 2.6*
	Cont	4.5 $\pm$ 0.6	19.0 $\pm$ 0.6	5.4 $\pm$ 0.3	29.1 $\pm$ 0.9	2.34 $\pm$ 0.10	9.1 $\pm$ 1.2	4.6 $\pm$ 0.7	24.6 $\pm$ 1.1
	DCA	4.6 $\pm$ 0.7	24.4 $\pm$ 1.2*	5.4 $\pm$ 0.4	32.7 $\pm$ 1.7	1.7 $\pm$ 0.05**	8.2 $\pm$ 0.8	10.8 $\pm$ 0.8**	21.9 $\pm$ 1.6*
1.0 mM lactate + 0.2 mM pyruvate	Cont	4.5 $\pm$ 0.6	15.5 $\pm$ 1.0	3.2 $\pm$ 0.5	22.2 $\pm$ 0.9	1.66 $\pm$ 0.31	7.6 $\pm$ 1.7	4.7 $\pm$ 0.9	18.5 $\pm$ 3.3
	Dex	6.4 $\pm$ 0.8*	17.6 $\pm$ 1.4	3.1 $\pm$ 0.6	27.6 $\pm$ 2.3*	1.51 $\pm$ 0.17	8.9 $\pm$ 1.4	5.6 $\pm$ 1.2	21.8 $\pm$ 2.3*
	Cont	5.0 $\pm$ 0.5	15.5 $\pm$ 0.7	4.4 $\pm$ 0.5	22.2 $\pm$ 0.9	1.35 $\pm$ 0.04	6.7 $\pm$ 0.5	4.5 $\pm$ 0.5	17.7 $\pm$ 1.2
	DCA	4.0 $\pm$ 0.2*	16.4 $\pm$ 1.0	4.3 $\pm$ 0.6	21.6 $\pm$ 1.0	1.33 $\pm$ 0.07	5.2 $\pm$ 0.2	6.9 $\pm$ 0.6*	14.7 $\pm$ 0.8*

Total pyruvate metabolism

In the control cells, the use of the more reduced substrate significantly diminished the total substrate utilization, the mean values for the combined controls being 29.7 ± 1.0 and 23.8 ± 1.4 ($P < 0.01$, $n = 12$) nmol/15 min per mg wet wt. with pyruvate alone and with a mixture of lactate plus pyruvate as the substrates respectively. Addition of dexamethasone resulted in a small stimulation of the total substrate utilized, which was only significant with 1.0 mM lactate/0.2 mM pyruvate as the substrate. The effect of dichloroacetate was substrate-dependent. With 1.0 mM pyruvate as the substrate there was a small increase in total pyruvate metabolism; however, increasing the redox state eliminated this stimulation.

Tricarboxylic acid cycle flux

Production of $^{14}\text{CO}_2$ from [3- 14 C]pyruvate is mainly the result of tricarboxylic acid cycling, although there is a small contribution from malic enzyme and PEPCK as a result of randomization of the carbon atoms and conversion of the tricarboxylic acid-cycle intermediates into glucose. The results in Table 3 indicate that there was an increased flux through the tricarboxylic acid cycle in control cells when the more oxidized substrate was employed, the respective fluxes being 5.74 ± 0.23 and 3.79 ± 0.40 ($n = 12$, $P < 0.001$) nmol/15 min per mg wet wt. The ratio of $^{14}\text{CO}_2$ production from the two labels, [3- 14 C]/[1- 14 C], was found to be 0.25, which compares favourably with the previous value of 0.21 determined in fasted rats with 2 mM lactate as the substrate [31]. Neither dexamethasone nor dichloroacetate had any significant effect on the flux through the cycle as measured by this method with either substrate.

Malic enzyme and PEPCK flux

In order to calculate PDH flux, it is necessary to correct the $^{14}\text{CO}_2$ released from [1- 14 C]lactate or pyruvate for $^{14}\text{CO}_2$ released during the PEPCK or malic enzyme reactions. This approach has been used previously to calculate PDH flux by Claus and Pilkis [31] and both PDH and pyruvate carboxylase fluxes by Agius and Alberti [32]. However, the former calculated the factor, *F*, to allow for recycling via pyruvate kinase from published data obtained under non-equivalent conditions, whereas the latter measured only the incorporation of $\text{NaH}^{14}\text{CO}_3$

into pyruvate and neglected the lactate component. From our experiments, with pyruvate alone as the substrate, neglecting lactate formation is likely to lead to an underestimate of the pyruvate kinase flux in control cells by 30%. This results in an underestimate of the flux through PEPCK and malic enzyme, and therefore an over- and under-estimate of the fluxes through PDH and pyruvate carboxylase respectively. Table 3 shows the *F* factor as calculated from the pyruvate kinase fluxes in Tables 1 and 2. Incubating the cells with dichloroacetate in the presence of 1.0 mM pyruvate as the substrate resulted in an 18% decrease in lactate plus pyruvate formation, the pyruvate kinase flux values being 12.5 ± 1.2 and 10.2 ± 0.5 nmol of [14 C]PEP incorporated/15 min per mg wet wt. of cells respectively for control and dichloroacetate-treated cells ($P < 0.01$, $n = 6$). This was reflected in a decrease in the *F* factor. When 1.0 mM lactate/0.2 mM pyruvate was used as the substrate, pyruvate kinase flux was greatly diminished; however, the effect of dichloroacetate was still apparent, the rates of pyruvate plus lactate formation being 5.6 ± 0.4 and 3.7 ± 0.6 nmol of [14 C]PEP incorporated/15 min per mg wet wt. of cells respectively for control and dichloroacetate-treated cells ($P < 0.01$, $n = 6$). As the rate of glucose synthesis was also inhibited by dichloroacetate in the presence of the more reduced substrate, the *F* factor remained unchanged.

The contribution of malic enzyme and PEPCK to $^{14}\text{CO}_2$ production from the 1- 14 C-labelled substrates is also shown in Table 3. It is evident that $^{14}\text{CO}_2$ released by malic enzyme and PEPCK makes a significant contribution to the $^{14}\text{CO}_2$ released from [1- 14 C]lactate plus pyruvate or [1- 14 C]pyruvate (the mean values being 46% and 44% respectively). This is comparable with that found by Claus and Pilkis [31]. The higher flux noted in control cells with pyruvate alone as the substrate would tend to support the conclusion that, with increasing cytosolic NADH/NAD $^+$ ratios, the malate/oxaloacetate equilibrium is shifted in favour of malate production, with less oxaloacetate available for the PEPCK reaction.

PDH and pyruvate carboxylase flux

Table 3 also shows the effects of dexamethasone treatment and dichloroacetate on PDH and pyruvate carboxylase flux. The use of the more reduced substrate tended to lower the flux through both enzymes; the respective values for the combined controls were 4.6 ± 0.5 and 5.2 ± 0.4 and 20.0 ± 1.7 and

24.6 ± 1.0 nmol/15 min per mg wet wt. of cells ($n = 12$) for J_{PDH} and J_{PC} with lactate plus pyruvate and pyruvate alone as the substrate respectively. This gives a pyruvate carboxylase/PDH ratio of 4.4 and 4.7 with the two substrates. These values for PDH are similar to those reported by Claus and Pilkis [31], but considerably less than those of Agius and Alberti [32]. However, as discussed above, the latter probably represents an overestimate of PDH, because pyruvate kinase flux was underestimated. Although no values for pyruvate carboxylase flux were calculated by Claus and Pilkis [31], these can be estimated from the data, giving a $J_{\text{PC}}/J_{\text{PDH}}$ ratio of approx. 5. This is comparable with the ratios reported in Table 3 and also with the pyruvate carboxylase/PDH ratio determined by using ^{13}C n.m.r. with alanine and ethanol as the substrate [42].

The method used for determination of pyruvate carboxylase and PDH fluxes assumes that all of the $^{14}\text{CO}_2$ -labelled oxaloacetate and malate are generated as a result of pyruvate carboxylase activity; however, Rognstad [43] has presented evidence in favour of some $^{14}\text{CO}_2$ incorporation by reverse PEPCK activity under gluconeogenic conditions with glutamine as the substrate. If a significant reverse PEPCK activity occurs under our conditions, then this would tend to decrease the specific radioactivity of the $[^{14}\text{C}]\text{PEP}$, underestimate the $[^{14}\text{C}]\text{-glucose}$ formed and increase the amount of radioactivity recovered as $^{14}\text{CO}_2$. This would result from incorporation of unlabelled CO_2 into C-4 of oxaloacetate by reverse PEPCK, randomization of the label at C-1 and C-4, and decarboxylation of the $^{14}\text{CO}_2$ at C-4 on recycling to PEP. Evidence that this does not introduce a significant error with physiological substrate concentrations can be derived from studies using 3-mercaptopicolinate, an inhibitor of PEPCK activity. If significant reverse PEPCK activity does occur, then the effect of the inhibitor would be to cause a proportionally greater decrease in the radioactivity lost as $^{14}\text{CO}_2$ than the decrease in $[^{14}\text{C}]\text{glucose}$. However, using 1.0 mM lactate plus 0.2 mM pyruvate as the substrate, we have shown that in the presence of the inhibitor the decrease in $^{14}\text{CO}_2$ output is equivalent to the decrease in the incorporation of radioactivity into glucose (results not shown). Similar results have also been shown during gluconeogenesis from 2 mM lactate in the perfused liver [40] and also during gluconeogenesis from 1 mM pyruvate in hepatocytes [44]. Similarly, as our calculations for PEPCK flux are derived from the product of the $[1\text{-}^{14}\text{C}]\text{-pyruvate}$ or -lactate incorporated into glucose and the F factor, then the net result of reverse PEPCK activity would be to underestimate $^{14}\text{CO}_2$ derived from PEPCK and overestimate PDH flux by an equivalent amount. Measurements of J_{PDH} with 1.0 mM lactate plus 0.2 mM pyruvate as the substrate in the presence of 0.1 mM 3-mercaptopicolinate have shown PDH flux to be independent of the inhibitor, the respective values being 6.3 ± 0.9 (4) and 7.7 ± 1.2 (4) nmol/15 min per mg wet wt. in the presence and absence of 3-mercaptopicolinate respectively. Similar results were found by Miles et al. [44] using 1.0 mM pyruvate, and suggest that the reverse PEPCK reaction does not introduce a significant error.

Although dichloroacetate had no significant effect on the oxidation of $[1\text{-}^{14}\text{C}]\text{lactate}$ plus pyruvate, Table 3 indicates that it increased PDH flux with both substrates, confirming that the increase in PDH_a measured in the isolated enzyme after dichloroacetate treatment is expressed in the intact cell [31,38,39]. The magnitude of the response depended on the substrate employed. With a mixture of lactate plus pyruvate, dichloroacetate increased J_{PDH} by 54%, where with pyruvate alone as the substrate there was a 135% increase in PDH flux. Addition of dichloroacetate had no significant effect on the J_{PC} with the more oxidized substrate, although it had a large effect on the par-

tititioning of pyruvate between PDH and pyruvate carboxylase, $J_{\text{PC}}/J_{\text{PDH}}$ being lowered from 5.4 to 2.1. In contrast, dichloroacetate significantly inhibited J_{PC} with a mixture of lactate and pyruvate as substrates. However, there was a similar decrease in the $J_{\text{PC}}/J_{\text{PDH}}$ ratio from 3.9 (control) to 2.1 (dichloroacetate). A number of possible explanations exist to explain decreased flux through pyruvate carboxylase. It has been suggested that the enzyme is inhibited by oxalate or glyoxalate as a result of metabolism of dichloroacetate [39]. This is unlikely under these conditions, as flux through the enzyme was not significantly inhibited with pyruvate as the substrate. The increase in mitochondrial NADH/NAD^+ ratio induced by the activation of PDH might inhibit the pyruvate carboxylase reaction. Control of pyruvate carboxylase by the NADH/NAD^+ ratio has been suggested from studies using mild inhibitors of the respiratory chain, possibly as a result of an increase in the matrix concentration of malate, which could act as an inhibitor of the enzyme [45]. A further possibility is that at physiological concentrations of substrate the supply of pyruvate for mitochondrial metabolism may become limiting. In the presence of dichloroacetate, the increased cytosolic redox state [31,41] would decrease the available pyruvate for transport into the mitochondria. This problem might not exist with pyruvate alone as the substrate, as the cytosolic pyruvate concentration would be considerably higher. This would be consistent with the inability of dichloroacetate to increase total pyruvate metabolism in the presence of lactate plus pyruvate (Table 3), there being competition between PDH and pyruvate carboxylase for the available matrix pyruvate.

Treatment of the rats and hepatocytes with dexamethasone caused a 20% decrease in PDH flux with 1.0 mM pyruvate as the substrate. This was accompanied by a similar percentage increase in pyruvate carboxylation, although the absolute increase was 5-fold that of the decrease in PDH. The $J_{\text{PC}}/J_{\text{PDH}}$ ratio increased from 4.2 to 6.3 after treatment with dexamethasone, indicating a diversion of pyruvate through to glucose. This effect would also be consistent with the previously reported increases in the mitochondrial acetyl-CoA/CoA and ATP/ADP ratios [7], although the decrease in glutamate measured after dexamethasone treatment may also be an important factor in increased flux through pyruvate carboxylase. In the presence of lactate plus pyruvate as the substrate, dexamethasone treatment resulted in a small non-significant increase in PDH flux and a smaller increase in pyruvate carboxylation, such that the $J_{\text{PC}}/J_{\text{PDH}}$ ratio remained unchanged (3.9 for control and for dexamethasone treatment). The difference between the effect of the steroid with the two substrates employed may be the result of two factors: first, the lower availability of cytosolic pyruvate may limit the ability of the steroid to stimulate pyruvate carboxylase, and second, an increased mitochondrial NAD^+/NADH ratio observed after dexamethasone treatment [46] may act to relieve the restraint on PDH.

Conclusion

In conclusion, the results indicate that dexamethasone increases the rates of both gluconeogenesis and pyruvate carboxylation in the intact cell. This confirms the conclusions drawn from studies using isolated mitochondria prepared from steroid-treated animals [16–20] and studies using control-strength analysis [8,9]. The percentage stimulation of pyruvate carboxylase flux is much less than that measured in intact mitochondria with non-physiological levels of substrate [16–20], indicating a probable enhancement of the response during the isolation of the organelle. Similarly, the large increase in pyruvate oxidation observed in isolated mitochondria prepared from steroid-treated animals

was not observed *in situ*, suggesting that it may be an artifact induced by the conditions used for mitochondrial isolation and incubation. The stimulation of gluconeogenesis after steroid treatment was independent of changes in J_{PK} , lactate and pyruvate formation being increased in parallel with the stimulation of glucose synthesis. Therefore the results do not support the conclusion that 50% of the stimulation of gluconeogenesis by steroid hormones can be attributed to an inhibition of pyruvate kinase [8,9]. Evidence for an inhibition of pyruvate kinase flux by dexamethasone in the latter studies was based on an increased sensitivity of gluconeogenesis to changes in oxaloacetate concentrations in the presence of dexamethasone. However, as the authors [8,9] point out, this is only indicative of an inhibition of pyruvate kinase if there are no effects of dexamethasone at either the steps between PEPCK and glucose or at PEPCK itself. It is evident from our studies that the partitioning of PEP at the branch point with pyruvate kinase is not significantly different in control and steroid-treated animals; thus it would seem unlikely that steps between PEP and glucose are altered by dexamethasone. In the absence of any evidence for effects of dexamethasone at the level of pyruvate kinase or the steps between PEP and glucose, the data of Sistare and Haynes [8,9,36] may be re-interpreted in terms of a stimulation at the level of PEPCK, either by an increased activity of the enzyme or by increased substrate supply, rather than an inhibition of pyruvate kinase. The stimulation of pyruvate carboxylase flux would suggest that an increased supply of substrate is a possible candidate for control; however, the percentage stimulation of J_{PC} was considerably less than the stimulation of the flux through PEPCK to glucose measured in this study, suggesting that control may be exerted at both sites. In view of the increased oxidative capacity of mitochondria isolated from animals treated with dexamethasone [47], the finding that PEPCK can be inhibited by physiological concentrations of 2-oxoglutarate may be important in this respect [48].

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