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Effect of fatty acids and ketones on the activity of pyruvate dehydrogenase in skeletal-muscle mitochondria

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1. The presence of palmitoyl-L-carnitine and acetoacetate (separately) decreased flux through pyruvate dehydrogenase in isolated mitochondria from rat hind-limb muscle. The effect of acetoacetate was dependent on the presence of 2-oxoglutarate and Ca^{2+}. 2. Palmitoylcarnitine, but not acetoacetate, also decreased the mitochondrial content of active dephospho-pyruvate dehydrogenase (PDH_A). This effect was large only in the presence of EGTA. Addition of Ca^{2+}–EGTA buffers stabilizing pCa values of 6.48 or lower gave near-maximal values of PDH_A content, irrespective of the presence of fatty acids or ketones when mitochondria were incubated under the same conditions used for the flux studies, i.e. at low concentrations of pyruvate. There was, however, a minor decrement in PDH_A content in response to palmitoylcarnitine oxidation when the substrate was L-glutamate plus L-malate. 3. Measurement of NAD^+, NADH, CoA and acetyl-CoA in mitochondrial extracts in general showed decreases in [NAD^+]/[NADH] and [CoA]/[acetyl-CoA] ratios in response to the oxidation of palmitoylcarnitine and acetoacetate, providing a mechanism for both decreased PDH_A content and feedback inhibition of the enzyme in the PDH_A form. However, only changes in [CoA]/[acetyl-CoA] ratio appear to underlie the decreased PDH_A content on addition of palmitoylcarnitine when mitochondria are incubated with L-glutamate plus L-malate (and no pyruvate) as substrate. 4. The effect of palmitoylcarnitine oxidation on flux through pyruvate dehydrogenase and on PDH_A content is less marked in skeletal-muscle mitochondria than in cardiac-muscle mitochondria. This may reflect the less active oxidation of palmitoylcarnitine by skeletal-muscle mitochondria, as judged by State-3 rates of O_2 uptake. In addition, Ca^{2+} concentration is of even greater significance in pyruvate dehydrogenase interconversion in skeletal-muscle mitochondria than in cardiac-muscle mitochondria.

It is well established that cardiac muscle preferentially oxidizes fatty acids and ketones rather than glucose, when these substrates are available (Randle *et al.*, 1963, 1964; Newsholme & Randle, 1964; Garland *et al.*, 1964*b*; Neely *et al.*, 1969; Neely & Morgan, 1974), and it is considered that this underlies the response of muscle tissue to the stress of starvation (Randle *et al.*, 1966). Part of the mechanism of this response involves an inhibition of flux through the pyruvate dehydrogenase reaction (EC 1.2.4.1, 2.3.1.12, 1.6.4.3), and this has been reproduced by perfusion of isolated rat hearts with palmitate, acetoacetate or acetate (Evans *et al.*, 1963; Garland *et al.*, 1964*a*; Randle *et al.*, 1970). The diminished flux through pyruvate dehydrogenase under these conditions can be attributed partly to end-product inhibition by elevated concentrations of NADH and acetyl-CoA (Garland & Randle, 1964; Bremer, 1969; Tsai *et al.*, 1973) and partly to a decreased content of PDH_A (Wieland *et al.*, 1971; Kerbey *et al.*, 1976; Olson *et al.*, 1978), the catalytically active dephosphorylated form of the enzyme (Linn *et al.*, 1969*a,b*). This change in the content of PDH_A arises from an increase in the activity of the pyruvate dehydrogenase kinase relative to that of the phosphatase, and is at least partly explained by the increase in [NADH]/[NAD^+] and [acetyl-CoA]/[CoA] ratios seen during fatty acid and ketone oxidation (Batenburg & Olson,

Abbreviation used: PDH_A, active (dephospho) pyruvate dehydrogenase.

1976; Kerbey *et al.*, 1976, 1977; Hansford, 1977). Acetyl-CoA and NADH have been shown to enhance kinase activity (Pettit *et al.*, 1975; Cooper *et al.*, 1975; Kerbey *et al.*, 1976), possibly through altering the degree of acetylation of the lipoic acid of the transacetylase subunit of the enzyme (EC 2.3.1.12; Cate & Roche, 1978). Attempts have been made to apportion quantitative significance to feedback control and to control by covalent modification (i.e. by changes in PDH_A content) in the overall response to fatty acid oxidation (Hansford & Cohen, 1978; Dennis *et al.*, 1979). The answer is very dependent on conditions, especially on the concentrations of pyruvate, ADP and Ca^{2+}, all of which tend to favour a high steady-state PDH_A content (Hansford, 1977; Hansford & Cohen, 1978). At the same time, increased availability of acetyl-CoA and NADH may not be the only mechanism whereby kinase activity is enhanced in the states of diabetes and starvation, as there is evidence of a modification of the kinase which is stable during the making of a mitochondrial preparation, conditions under which metabolite ratios *in vivo* are not expected to be preserved (Hutson & Randle, 1978; Kerbey & Randle, 1981).

Since the mass of voluntary muscle is much greater than that of cardiac muscle, the physiological significance of the sparing of glucose in muscle tissues is quite dependent on the relevance, or otherwise, of these results to skeletal muscle. Here, the experimental results are less clear (see Berger *et al.*, 1976, for a review of the literature). However, there is good evidence that the oxidation of lactate by the isolated hindquarter of the rat is markedly decreased in starvation and diabetes (Berger *et al.*, 1976). The same effect is generated by perfusion with acetoacetate, though not with octanoate or palmitate (Berger *et al.*, 1976). Consistent with this is the finding by the same group (Hagg *et al.*, 1976) that PDH_A content of hindquarter muscle is decreased by starvation and diabetes, as also found by Hennig *et al.* (1975), and that this can also be reproduced by perfusion with acetoacetate. Significantly, the latter effect was abolished on electrical stimulation of the muscle.

The aims of the present work were fourfold. Firstly, we wanted to extend the work of Hagg *et al.* (1976) to include a study of the effects of long-chain fatty acids on the PDH_A content of isolated skeletal-muscle mitochondria, as such an effect seemed a possible mechanism of the marked decrease in lactate oxidation seen in starvation (Berger *et al.*, 1976), despite the failure of palmitate to inhibit lactate oxidation in resting skeletal muscle (Pearce & Connett, 1980). It seemed likely that the mitochondrial incubation conditions, especially the availability of Ca^{2+} and ADP (Hansford, 1977; Hansford & Cohen, 1978) would be crucial to the poise of the interconversion system and thus to the demonstration of any putative effects of palmitate oxidation. Secondly, we wished to study the relative importance of feedback inhibition and enzyme interconversion in any response of pyruvate oxidation to fatty acids and ketones. This can be done by comparing flux through the pyruvate dehydrogenase reaction with PDH_A content under identical conditions and is an extension of work that we have previously done with heart mitochondria (Hansford & Cohen, 1978). Thirdly, we wished to characterize the response of the system to Ca^{2+} ions, as extramitochondrial Ca^{2+} concentrations in the range pCa 6–7 have profound effects on the steady-state PDH_A content of isolated heart mitochondria (Hansford & Cohen, 1978; Denton *et al.*, 1980; Hansford, 1981), and such a mechanism might underlie the marked effect of exercise in attenuating the response of muscle PDH_A content to acetoacetate perfusion (Hagg *et al.*, 1976). Finally, we wished to establish baseline values for young adult animals with a view to subsequent examination of these processes in senescent animals. It has previously been shown that fatty-acylcarnitine is less actively oxidized by heart mitochondria from senescent rats (Chen *et al.*, 1972; Hansford, 1978), and were such an effect true of skeletal muscle it could have physiological significance in the less-effective sparing of glucose for the brain in old age.

Materials and methods

Mitochondrial preparations

Most of the hind-limb muscle was removed from one male Sprague–Dawley rat (300–400 g), trimmed of fat and connective tissue and cut finely with scissors in ice-cold modified Chappell–Perry (CP) medium (Chappell & Perry, 1954; Mickelson *et al.*, 1980), comprising 0.1 M-KCl, 0.05 M-Tris/HCl, pH 7.5, 5 mM-$MgCl_2$, 1 mM-ATP, 1 mM-EGTA and 0.5% (w/v) bovine serum albumin. The washed muscle was incubated with Nagarse for 30 min at 0°C, at a concentration of 1 g of muscle and 1 mg of Nagarse/10 ml of CP medium. During this period the tissue was repeatedly homogenized by hand in a Teflon Dounce homogenizer. The resulting homogenate was diluted with CP medium to 30 ml/g of muscle, filtered through two layers of cheesecloth, and centrifuged at 600 ***g*** for 10 min. The supernatant was decanted, again filtered through cheesecloth, and centrifuged at 10000 ***g*** for 10 min. The mitochondrial pellets were combined and resuspended in 10 ml of 0.25 M-sucrose/10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] (potassium salt)/1 mM-EGTA, pH 7.2, and then centrifuged at 10000 ***g*** for 10 min. The pale fluffy layer of the resulting pellet was removed by gentle swirling, and this centrifugation step was

repeated. The final pellet was resuspended in sucrose/Hepes/EGTA medium at 20–25mg of protein/ml. The final yield was approx. 3mg of mitochondrial protein/g of muscle.

Where flux through pyruvate dehdyrogenase was measured in the absence of Ca^{2+}, 3mM-NaCl was included in the sucrose/Hepes/EGTA medium, to facilitate the withdrawal of mitochondrial Ca^{2+} by EGTA.

Mitochondrial incubations

O_2 uptake was measured at 25°C with a Clark-type O_2 electrode (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.) in a 2ml glass vessel (Gilson). The composition of the medium is given in the appropriate Figure or Table.

PDH_A content was measured as described previously (Hansford, 1976, 1977, 1981). Briefly, 50μl samples of mitochondrial incubation were withdrawn and added to 250μl of a 'stop' solution designed to prevent further enzyme interconversion. Determination of PDH_A content was then based on the measurement of NAD^+ reduction, under $V_{max.}$ conditions. Care was taken to begin the spectrophotometric determination of activity within 2min of quenching of interconversion: under these conditions loss of enzyme in the 'stop' solution is trivial. When mitochondria were prepared as described, contamination with lactate dehydrogenase or NADH oxidase was quantitatively insignificant, though always investigated.

Ca^{2+}–EGTA buffers were made as described by Portzehl *et al.* (1964). In experiments in which Ca^{2+}–EGTA buffers were used, the pH of the incubation medium and of all additions was meticulously adjusted to 7.20.

Measurement of flux through pyruvate dehydrogenase

Flux was measured at low concentrations (approx. 60μM) of pyruvate by using the pyruvate-regenerating system described by Davis-van Thienen & Davis (1981). The sorbitol dehydrogenase is not active enough to maintain a true equilibrium, a drawback recognized by the original authors. However, use of a range of sorbitol dehydrogenase activities (0.5–2.0units/ml), with the larger amounts used when ADP-stimulated respiration was studied, allowed final (5min) pyruvate concentrations within the range 57–68μM in all mitochondrial experiments, and this was judged acceptable. The flux determinations depended on knowledge of the pool of pyruvate plus lactate: the isotopically labelled compound was added with the other components of the incubation, as given in the Table legend, 30min before the addition of the mitochondria. Controls indicated complete equilibrium at this time, at 25°C. Mitochondrial incubations were performed in 25ml Erlenmeyer flasks, sealed with serum caps. The reaction was terminated by the injection of 0.5ml of 16% (v/v) $HClO_4$, after which the flasks were shaken at 37°C for 1h to allow trapping of CO_2 by 0.2ml of 1M-Hyamine hydroxide solution in the centre well. $^{14}CO_2$ was determined by a standard scintillation-counting technique.

Extraction of mitochondria and measurement of coenzymes

Samples (1ml) of the mitochondrial incubation were removed and extracted with $HClO_4$, for determination of NAD^+, CoA and acetyl-CoA, or with ethanolic KOH, for determination of NADH. Extraction techniques and assay methods are described by Hansford (1976). A slight modification was that NAD^+ and NADH were measured with the dual-wavelength spectrophotometer (Aminco–Chance DW2A) instead of by fluorimetry.

Enzymes and reagents

Enzymes were purchased from Boehringer Mannheim Corp., New York, NY, U.S.A., with the exception of Nagarse, which was from Enzyme Development Corp., New York, NY, U.S.A. Sodium [1-^{14}C]pyruvate was from The Radiochemical Centre, Amersham/Searle Corp., and palmitoyl-L-carnitine was from P-L Biochemicals. All other reagents were of the highest quality commercially available and were dissolved in deionized and glass-distilled water.

Results

Evaluation of the skeletal-muscle mitochondrial preparation

Table 1 presents a profile of the oxidative capacity of this preparation, as skeletal-muscle mitochondria are not very frequently studied. Absolute rates of O_2 uptake are higher than those presented previously (see e.g., Azzone & Carafoli, 1960; Chen *et al.*, 1972; Van Hinsbergh *et al.*, 1978), and the respiratory control ratios are somewhat higher, too. Germane to the present study is the fact that these mitochondria oxidize palmitoylcarnitine more actively than did those studied previously (Pande & Blanchaer, 1971; Chen *et al.*, 1972; Van Hinsbergh *et al.*, 1978), but this rate is still well short of that achieved with pyruvate or glutamate plus malate, in distinction to results obtained with heart mitochondria (Hansford, 1978, 1983). Our mitochondrial preparation used essentially all of the hind-limb muscle, and thus was derived from both red and white fibres. Pande & Blanchaer (1971) have shown that mitochondria from red muscles oxidize fatty acid more actively than do those from white

Table 1. *O_2 uptake by rat skeletal-muscle mitochondria incubated in the presence of various substrates*
Mitochondria (0.5–1 mg of protein) were added to 2 ml of medium comprising 0.13 M-KCl, 20 mM-Hepes (potassium salt), 5 mM-potassium phosphate and the appropriate substrate, at the concentration indicated. The pH was 7.2 and the temperature was 25°C. O_2 uptake was measured with an O_2 electrode, as described in the Materials and methods section. State-3 rates (see Chance & Williams, 1956) were elicited by the addition of ADP (final concn. 1 mM) 2 min after the addition of the mitochondria: State-4 rates were measured after the cessation of ADP phosphorylation. Data are presented as means ± S.E.M. for the numbers of preparations in parentheses.

Substrate	Rate of O_2 uptake (ng-atoms of O/min per mg of protein)		Respiratory control ratio
	State 3	State 4	
Pyruvate (2.5 mM) + L-malate (1 mM)	445 ± 15 (6)	47 ± 8 (6)	10.6
Acetyl-L-carnitine (5 mM) + L-malate (1 mM)	215 ± 11 (6)	72 ± 9 (6)	3.3
Palmitoyl-L-carnitine (20 μM) + L-malate (1 mM)	313 ± 12 (6)	40 ± 4 (6)	8.3
Glutamate (4 mM) + L-malate (5 mM)	447 ± 20 (4)	42 ± 5 (4)	11.1
Pyruvate (2.5 mM) + L-glutamate (4 mM) + L-malate (5 mM)	506 ± 34 (3)	45 ± 5 (3)	11.5
Palmitoyl-L-carnitine (20 μM) + L-glutamate (4 mM) + L-malate (5 mM)	538 ± 40 (3)	36 ± 3 (3)	15.5
2-Oxoglutarate (0.5 mM) + L-malate (1 mM)	131 ± 5 (6)	47 ± 4 (6)	2.9
2-Oxoglutarate (0.5 mM) + L-malate (1 mM) + acetoacetate (1 mM)	379 ± 10 (3)	39 ± 3.6 (3)	5.7

muscles: the present activities (Table 1) are higher than those reported by Pande & Blanchaer (1971) from either source, but, consistent with their findings, the activity of palmitoylcarnitine oxidation expressed as a fraction of the activity of pyruvate oxidation (0.7) falls between their values for red (0.84) and white (0.47) muscle. Acetoacetate is well oxidized (Table 1) when non-saturating concentrations of 2-oxoglutarate are available as a source of succinyl-CoA, which allows formation of acetoacetyl-CoA.

Effect of extramitochondrial pCa on PDH_A content

The interconversion of cardiac pyruvate dehydrogenase is quite sensitive to the free Ca^{2+} concentration of the medium to which the mitochondria are exposed (Hansford & Cohen, 1978; Denton *et al.*, 1980; Hansford, 1981). Fig. 1 presents the results of experiments using skeletal-muscle mitochondria in which the Ca^{2+} concentration (expressed as pCa) of the medium was systematically varied with Ca^{2+}–EGTA buffers (Portzehl *et al.*, 1964); 50% activation of pyruvate dehydrogenase occurs at approx. pCa 6.60. These incubations were sampled at 5 min, at which point interconversion had substantially achieved a steady state: a separate study in which sampling was conducted at 10 min gave 50% activation at pCa 6.80. These values are slightly higher than those found for cardiac mitochondria (Hansford, 1981), incubated at the same Na^+ (10 mM) and Mg^{2+} (1 mM) concentrations, suggesting that skeletal-muscle mitochondria are capable of withdrawing Ca^{2+} from lower extramitochondrial free Ca^{2+} concentrations. More experiments would be needed

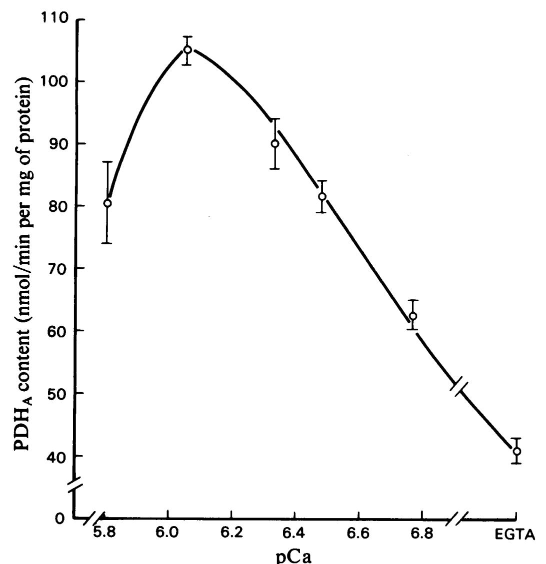

Fig. 1. *Effect of extramitochondrial pCa on PDH_A content of skeletal-muscle mitochondria*
Mitochondria (0.5–1 mg of protein) were added to 1 ml of medium comprising 0.13 M-KCl, 20 mM-Hepes (potassium salt), 10 mM-NaCl, 1 mM-$MgCl_2$, 5 mM-potassium phosphate, 4 mM-L-glutamate, 5 mM-L-malate and containing a Ca^{2+}–EGTA buffer of the indicated pCa (2 mM as EGTA). The pH was 7.2 and the temperature was 25°C. Duplicate portions of the incubation were removed at 5 min, quenched and assayed for PDH_A content as described in the Materials and methods section. Error bars represent the S.E.M. for values from four incubations, involving two separate mitochondrial preparations.

for proof. Relevant to the experiments discussed below is the fact that pCa 6.05 gives maximal PDH_A values [though slightly elevated State-4 rates of O_2 uptake (results not shown); see Chance & Williams (1956) for terminology], whereas pCa 6.48 gives more than 50%, but not maximal, activation. It is noted that changes in pCa in the medium do not affect the total PDH, as opposed to PDH_A, content of these mitochondria. This was demonstrated by the near-correspondence of values for PDH_A content obtained in the presence of 2.5mM-pyruvate and 0.125mM-dichloroacetate and at pCa values 6.05 and >8. These values were 78 ± 2 and 72 ± 4 nmol/min per mg of protein respectively and could not be further elevated by higher concentrations of dichloroacetate, a potent inhibitor of pyruvate dehydrogenase kinase (Whitehouse *et al.*, 1974). They thus arguably correspond to complete conversion into the PDH_A form. In addition, effects of pCa on PDH_A content are fully reversible by EGTA, as noted previously for the cardiac system (Hansford & Cohen, 1978).

Effect of palmitoylcarnitine and acetoacetate on PDH_A content of mitochondria oxidizing glutamate plus malate or 2-oxoglutarate

Table 2 represents the results of early experiments designed to investigate whether the oxidation of acetoacetate or palmitate affects pyruvate dehydrogenase interconversion. Palmitate was presented as palmitoyl-L-carnitine, in the presence of an optimal amount of bovine serum albumin. It clearly had no effect when EGTA was present: in this case (Table 2, column 2), the low steady-state PDH_A content is evidence for a relative excess of kinase activity over phosphatase activity. Phosphatase activity would be expected to be lowered by Ca^{2+} withdrawal from the mitochondria (Denton *et al.*, 1972; Pettit *et al.*, 1972; Randle *et al.*, 1974; Severson *et al.*, 1974). When the kinase was also inhibited, by the inclusion of dichloroacetate (Whitehouse *et al.*, 1974), the poise of kinase and phosphatase activity was more favourable for the demonstration of an effect of palmitoylcarnitine oxidation, and indeed a large decrease in PDH_A content was observed (Table 2, column 3): controls indicated that this was not due to the presence of the albumin alone. Acetylcarnitine oxidation was also effective, although it is not oxidized as actively as palmitoylcarnitine (Table 1), and thus would not be expected to be such an effective donor of hydrogen and acetyl groups (see below). By contrast, acetoacetate oxidation had no significant effect on PDH_A content. This may reflect inadequate activation of acetoacetate owing to the low 2-oxoglutarate dehydrogenase activity, and therefore generation of succinyl-CoA, to be expected at 0.5mM-2-oxoglutarate, in the near-absence of Ca^{2+} (McCormack & Denton, 1979). Notably, palmitoylcarnitine oxidation gave marked inactivation of pyruvate dehydrogenase when the substrate was 0.5mM-2-oxoglutarate plus 0.5mM-L-malate, in the presence of EGTA and dichloroacetate. Thus, in a separate series of experiments, PDH_A content was found to be 81 ± 1 (4) nmol/min per mg of protein in the absence of palmitoylcarnitine, but only 12 ± 7 (4) nmol/min per mg of protein in its presence. Thus inactivation of pyruvate dehydrogenase is fully possible under these experimental conditions, and the poor activation of acetoacetate inferred above becomes the more likely as a reason for its ineffectiveness in decreasing PDH_A content in Table 2.

When phosphatase activity was potentiated by Ca^{2+} (Table 2, column 4), PDH_A contents were near-maximal throughout, with the presence of

Table 2. *Effect of palmitoylcarnitine and acetoacetate oxidation on the PDH_A content of skeletal-muscle mitochondria*

Mitochondria (0.5–1 mg of protein) were added to 2ml of a medium comprising (*a*) 0.13M-KCl, 20mM-Hepes (potassium salt), 10mM-NaCl, 1mM-$MgCl_2$, 5mM-potassium phosphate, 5mM-L-malate and 4mM-L-glutamate, or (*b*) the same medium but with 0.5mM-2-oxoglutarate and 0.5mM-L-malate replacing the glutamate and 5mM-malate. The pH was 7.2 and the temperature was 25°C. Additional oxidizable substrates were present as indicated below: when palmitoyl-L-carnitine was added, bovine serum albumin was included at 2.5mg/ml. In addition, each incubation contained 1mM-EGTA, or 1mM-EGTA plus 125 μM-dichloroacetate (DCA), or a Ca^{2+}–EGTA buffer (1mM as EGTA) stabilizing pCa 6.05. The incubations were sampled for PDH_A content 10min after the addition of the mitochondria. Values given are means ± S.E.M., with the numbers of mitochondrial preparations in parentheses. *Indicates a significant ($P < 0.05$) effect of acylcarnitine.

	PDH_A content (nmol/min per mg of protein)		
Additional substrate	EGTA	EGTA + DCA	pCa 6.05
(*a*) None	43.9 ± 2.9 (10)	107 ± 6 (5)	102 ± 3 (11)
Palmitoyl-L-carnitine (50 μM)	42.6 ± 3.1 (11)	41.0 ± 2.2* (6)	80.3 ± 3.0* (6)
Acetyl-L-carnitine (1 mM)	46.8 ± 3.5 (6)	52.6 ± 1.8* (6)	85.9 ± 4.1* (6)
(*b*) None	34.9 ± 2.9 (6)	101 ± 6 (5)	94.8 ± 5.4 (7)
Acetoacetate (1 mM)	35.4 ± 3.0 (5)	95.9 ± 5.0 (5)	85.5 ± 6.3 (9)

acylcarnitine having only a slight (though statistically significant) effect. It is clear from these results (Table 2) that the availability of Ca^{2+} is of paramount importance in determining the relative activities of kinase and phosphatase and thus whether the modulating effects of fatty acid oxidation are observable.

One mechanism of such an effect of acylcarnitine oxidation is an alteration in the ratios [CoA]/[acetyl-CoA] and $[NAD^+]/[NADH]$ (Kerbey *et al.*, 1976, 1977; Hansford, 1977), and this is examined in Table 3. Clearly changes in $[NAD^+]/[NADH]$ ratio are not a plausible mechanism for the changes in PDH_A content detailed in Table 2. Thus the addition of palmitoylcarnitine only changes the $[NAD^+]/[NADH]$ ratio in the presence of EGTA, conditions in which there is no corresponding effect on PDH_A content (Table 2). It has no effect on $[NAD^+]/[NADH]$ ratio in the presence of EGTA plus dichloroacetate, conditions under which PDH_A content is markedly decreased by palmitoylcarnitine (Table 2). By contrast, large decreases in [CoA]/[acetyl-CoA] ratio are seen as a consequence of palmitoylcarnitine oxidation, in conditions under which a decreased PDH_A content is also seen, i.e. the use of a Ca^{2+}–EGTA buffer, or the presence of dichloroacetate. The description of the change in this effector ratio is limited only by the difficulty of detection of CoA during acylcarnitine oxidation. Thus under these conditions it is plausible that the predominant effect of fatty acid oxidation is to decrease the [CoA]/[acetyl-CoA] ratio and thus activate the pyruvate dehydrogenase kinase (Pettit *et al.*, 1975; Cooper *et al.*, 1975; Kerbey *et al.*, 1976), leading to a lowered steady-state content of PDH_A.

In the experiments of Tables 2(*b*) and 3(*b*), the picture that emerges is quite different, with the PDH_A content being quite unresponsive to a large decrease in the [CoA]/[acetyl-CoA] ratio caused by the introduction of acetoacetate (at pCa 6.48). Here, the $[NAD^+]/[NADH]$ ratio is very high in all experiments (cf. Table 3*a*), a consequence of the low activity of 2-oxoglutarate dehydrogenase in the presence of 0.5M-2-oxoglutarate. Thus the lipoic acid of the transacetylase subunits may be only slightly acetylated in the presence of acetoacetate, despite the low [CoA]/[acetyl-CoA] ratio, and this would be consistent with only modest kinase activity, certainly on the mechanism proposed by Cate & Roche (1978).

Acetoacetate is only effective in donating acetyl groups (i.e. lowering the [CoA]/[acetyl-CoA] ratio) when Ca^{2+} is present (cf. pCa 6.48 and EGTA). This may reflect the activation of 2-oxoglutarate dehydrogenase by Ca^{2+} (McCormack & Denton, 1979; Hansford & Castro, 1981), giving a more active provision of succinyl-CoA for the activation of acetoacetate. Secondly, the pCa value adopted to give optimal pyruvate dehydrogenase activation was not the same for Tables 2 and 3, although other conditions were identical. This prevents an exact description of the role of the effector ratios in modulating interconversion in these experiments, though the directions of change identified above are considered reliable. Elsewhere in this paper, pCa 6.48 was used when activation by Ca^{2+} was desired.

Table 3. *Effect of palmitoylcarnitine (a) and acetoacetate (b) on the [CoA]/[acetyl-CoA] and $[NAD^+]/[NADH]$ ratios of mitochondria oxidizing glutamate plus malate (a) or 2-oxoglutarate (b)*

Mitochondria were added to the appropriate medium described for Table 2, but at 0.5–1 mg of protein/ml. Samples (1 ml) were removed at 5 min and extracted with $HClO_4$ or ethanolic KOH. Details of the extraction procedure and the subsequent assay of mitochondrial coenzymes are given in the Materials and methods section. Data presented are mean values ± S.E.M. from at least three mitochondrial preparations. Abbreviation: ND, not detectable.

Conditions	CoA (nmol/mg of protein)	Acetyl-CoA (nmol/mg of protein)	[CoA]/[acetyl-CoA]	NAD^+ (nmol/mg of protein)	NADH (nmol/mg of protein)	$[NAD^+]/[NADH]$
(*a*) EGTA	0.41 ± 0.02	0.22 ± 0.04	1.74	1.73	1.59	1.09
pCa 6.48	0.42 ± 0.07	0.19 ± 0.04	2.31	0.88 ± 0.18	2.05 ± 0.05	0.43
EGTA + DCA	1.00 ± 0.11	0.08 ± 0.02	15	0.92 ± 0.20	1.69 ± 0.17	0.48
EGTA + palmitoylcarnitine	0.25	0.47	0.53	1.14	1.91	0.59
pCa 6.48 + palmitoylcarnitine	ND	0.09 ± 0.02	–	0.93 ± 0.12	2.09 ± 0.28	0.44
EGTA + DCA + palmitoylcarnitine	ND	0.08 ± 0.02	—	0.87 ± 0.15	1.79 ± 0.16	0.48
(*b*) EGTA	0.31 ± 0.09	0.03 ± 0.01	11.9	1.35 ± 0.05	0.20 ± 0.04	7.5
pCa 6.48	0.12 ± 0.02	0.11 ± 0.03	1.8	1.84 ± 0.20	0.42 ± 0.07	5.0
EGTA + DCA	0.55 ± 0.13	0.10 ± 0.02	5.7	2.27 ± 0.37	0.23 ± 0.06	14
EGTA + acetoacetate	0.34 ± 0.07	0.03 ± 0.002	11.3	1.37 ± 0.04	0.25 ± 0.06	6.3
pCa 6.48 + acetoacetate	0.05 ± 0.01	0.39 ± 0.08	0.15	1.46 ± 0.22	0.56 ± 0.06	2.7
EGTA + DCA + acetoacetate	0.25 ± 0.06	0.04 ± 0.01	6.1	1.93 ± 0.32	0.31 ± 0.04	7.1

Effect of palmitoylcarnitine and acetoacetate on flux through pyruvate dehydrogenase

The experiments described in the previous section were designed to investigate the effect of fatty acid and ketone oxidation on pyruvate dehydrogenase interconversion. To this end, the stable respiration, and hence [ATP]/[ADP] and [NAD+]/[NADH] ratios, characteristic of the oxidation of glutamate plus malate were an advantage. However, in the animal the question of significance is whether oxidation of fatty acids and ketones inhibits flux through pyruvate dehydrogenase. This requires experimental systems containing pyruvate, at a physiologically appropriate concentration. Notably, it was thought important to avoid supra-physiological concentrations of pyruvate (in excess of 0.15mM, as determined by Berger *et al.*, 1976), which have been shown in heart mitochondria to give complete interconversion of pyruvate dehydrogenase into the PDH_A form, regardless of the concentrations of other effectors (Hansford, 1977). This is considered to be a consequence of the inhibition of the kinase by pyruvate (Hucho *et al.*, 1972). Table 4 presents a study in which pyruvate concentrations were stabilized at approx. 60μM by using a novel system developed by Davis-van Thienen & Davis (1981), which combines the lactate dehydrogenase and sorbitol dehydrogenase reactions. Flux was measured by release of $^{14}CO_2$ from [1-^{14}C]pyruvate, at equilibrium with a large pool of lactate. The progress of the reaction was linear for the 5min period studied. The flux is proportional to O_2 uptake when the pyruvate-generating system provides the sole substrate. The introduction of palmitoylcarnitine gave an approx. 40–45% decrease in flux throughout the range of respiratory states (i.e. State 4, 50% State 3 and 80% State 3) when a Ca^{2+}–EGTA buffer is present (Table 4*a*), though a somewhat lesser inhibition of flux during resting (State 4) respiration in the absence of Ca^{2+} (Table 4*b*). Acetoacetate addition also resulted in a decreased flux through pyruvate dehydrogenase, with the decrease being largest in State 4 (Table 4*a*). When the mitochondria were depleted of Ca^{2+} by EGTA, in the presence of Na^+, acetoacetate addition gave no such inhibition in flux (Table 4*b*).

The effect of palmitoylcarnitine and acetoacetate oxidation on flux through pyruvate dehydrogenase (Table 4) could derive from an altered PDH_A content, an altered degree of feedback inhibition of PDH_A, or both. The studies reported in Table 5 were designed to evaluate the significance of changes in PDH_A content. The substrate was 60μM-pyruvate, in distinction from the experiments described above (Table 2), which used glutamate plus malate. Under these conditions of low pyruvate concentration, the presence of palmitoylcarnitine or acetoacetate was

Table 4. *Effect of palmitoylcarnitine and acetoacetate on flux through the pyruvate dehydrogenase reaction*

(*a*) Mitochondria (0.5–1 mg of protein) were added to flasks containing 1 ml of a medium comprising 0.105M-KCl, 20mM-Hepes (potassium salt), 10mM-NaCl, 1mM-$MgCl_2$, 10mM-potassium phosphate, 25mM-L-lactate, 25mM-fructose, 6mM-sorbitol, 1mM-NAD^+, 0.5–2 units of sorbitol dehydrogenase/ml, 20 units of lactate dehydrogenase/ml and a Ca^{2+}–EGTA buffer of pCa 6.48 and of EGTA concn. 1.5mM. Radiolabelled pyruvate or L-lactate had previously been added to allow equilibration and a final specific radioactivity of the lactate plus pyruvate pool of 0.05μCi/μmol (see the Materials and methods section for more details). In the incubations marked '50% State 3' or '80% State 3', 10mM-D-glucose and 1mM-ATP were additionally present, and hexokinase was added, immediately after the mitochondria, at an activity sufficient to generate either 50% or 80% of the respiratory rate achieved with saturating amounts of hexokinase, and the same incubation medium. (*b*) Conditions were exactly the same, except that 1mM-EGTA replaced the Ca^{2+}–EGTA buffer, and that the mitochondria had been pre-depleted of Ca^{2+} (see the Materials and methods section). In all cases, incubations were for 5min at 25°C. They were terminated with $HClO_4$ and flux was determined from $^{14}CO_2$ release, as described in the Materials and methods section. Values are presented as means ± S.E.M., with the numbers of incubations in parentheses. Each value is derived from incubations with at least three mitochondrial preparations. *Indicates a significant ($P < 0.05$) effect of palmitoylcarnitine or acetoacetate.

	Flux (nmol of $^{14}CO_2$/min per mg of protein)		
Additional substrate	State 4	50% State 3	80% State 3
(*a*) None	16.4 ± 2.1 (8)	47.3 ± 9.0 (6)	71.6 ± 4.7 (9)
Palmitoyl-L-carnitine (50μM)	10.1 ± 1.7* (8)	26.5 ± 5.2 (6)	38.9 ± 2.6* (9)
2-Oxoglutarate (0.5mM)	14.8 ± 2.1 (7)	38.2 ± 6.2 (8)	56.2 ± 3.0 (8)
2-Oxoglutarate (0.5mM) + acetoacetate (1mM)	9.5 ± 1.5* (7)	25.9 ± 4.7 (8)	41.2 ± 2.9* (8)
(*b*) None	21.1 ± 0.7 (8)	54.6 ± 1.8 (8)	
Palmitoyl-L-carnitine (50μM)	17.9 ± 0.6* (6)	26.9 ± 0.6* (8)	
2-Oxoglutarate (0.5mM)	21.9 ± 0.8 (7)	48.0 ± 1.0 (8)	
2-Oxoglutarate (0.5mM) + acetoacetate (1mM)	23.0 ± 0.7 (8)	47.5 ± 1.0 (8)	

Table 5. *Effect of palmitoylcarnitine and acetoacetate oxidation on the PDH_A content of mitochondria incubated at a low concentration (60 μM) of pyruvate*

(*a*) Mitochondria (0.5–1 mg of protein) were added to a medium comprising 0.13 M-KCl, 20 mM-Hepes (potassium salt), 10 mM-NaCl, 1 mM-$MgCl_2$, 5 mM-potassium phosphate, 1 mM-L-malate and containing a Ca^{2+}–EGTA buffer of pCa 6.48 (1 mM as EGTA). The pH was 7.2 and the temperature was 25°C. Pyruvate was present at 60 μM and was maintained approximately constant in concentration by supplementation with small volumes of 2 mM-pyruvate each time when 5 or 10% of the total O_2 in the system was depleted (see Hansford & Cohen, 1978). Assay of the medium at the end of the experiment indicated deviations of less than ±10% from 60 μM. Where indicated, 2-oxoglutarate, acetoacetate and palmitoyl-L-carnitine were additionally present at 0.5 mM, 1 mM and 50 μM respectively. Generation of enhanced rates of O_2 uptake, equal to 50% and 80% of State 3, was as described for Table 4. The incubations were sampled at 5 min, quenched and assayed for PDH_A content as described in the Materials and methods section. Results are mean values ± S.E.M. from eight experiments, with four different mitochondrial preparations. (*b*) The same protocol was used, except that all of the incubations were in State 4 (no hexokinase, ATP or glucose added) and that media contained either EGTA (1 mM) or EGTA (1 mM) plus dichloroacetate (DCA; 125 μM). Data are mean values ± S.E.M. from six determinations, involving six mitochondrial preparations.

	PDH_A content (nmol/min per mg of protein)		
Additional substrate	State 4	50% State 3	80% State 3
(*a*) None	91.4 ± 5.9	92.9 ± 6.0	99.2 ± 6.0
Palmitoylcarnitine	83.5 ± 3.3	83.9 ± 2.1	92.9 ± 4.4
2-Oxoglutarate	100 ± 4	100 ± 6	103 ± 4
2-Oxoglutarate + acetoacetate	103 ± 4	97.2 ± 5.3	104 ± 3

	PDH_A content in State 4 (nmol/min per mg of protein)	
Additional substrate	EGTA	EGTA + DCA
(*b*) None	33.0 ± 1.1	40.5 ± 2.0
Palmitoylcarnitine	18.0 ± 0.8	21.2 ± 1.2
2-Oxoglutarate	38.7 ± 1.5	45.3 ± 1.7
2-Oxoglutarate + acetoacetate	35.2 ± 2.1	39.7 ± 1.9

without effect on PDH_A content, which remained maximal or near-maxinal, provided that Ca^{2+} was present (pCa 6.48; Table 5*a*). When Ca^{2+} was removed with EGTA (Table 5*b*) there was a 45% decrease in PDH_A content owing to palmitoylcarnitine oxidation, but no effect of acetoacetate oxidation. Repetition of these experiments in the presence of dichloroacetate, to give a model system perhaps more responsive to fatty acid oxidation, gave slightly higher values of PDH_A content, with a 50% decrease with palmitoylcarnitine (Table 5*b*). This experiment was only performed in the absence of Ca^{2+}. Palmitoylcarnitine did not exert its effects through changing the total PDH content of the mitochondria in these experiments, as the decrease in PDH_A content owing to the addition of palmitoylcarnitine could be fully reversed by a subsequent addition of a Ca^{2+}–EGTA buffer (results not shown). These experiments used a lower initial concentration of EGTA.

The decrease in PDH_A content seen as a consequence of palmitoylcarnitine oxidation in the presence of EGTA (Table 5*b*) may provide the mechanism for the decreased flux through pyruvate dehydrogenase under these conditions (Table 4*b*). Indeed, comparison of PDH_A content and flux suggests little role for feedback inhibition under these conditions (State 4, EGTA). However, the PDH_A values in Table 5(*a*) provide no mechanism for the effect of fatty acid and ketone oxidation on flux in the presence of Ca^{2+} (Table 4*a*). This suggests that changes in feedback inhibition are predominant under these conditions, and this is examined in Table 6.

Introduction of palmitoylcarnitine decreased the $[NAD^+]/[NADH]$ ratio, when State-4 respiration in the presence of a Ca^{2+}–EGTA buffer (pCa 6.48) was studied (Table 6*a*). Introduction of acetoacetate decreased both $[NAD^+]/[NADH]$ and [CoA]/[acetyl-CoA] ratios. These changes are consistent with the decreases in flux seen under these conditions (Table 4*a*), on the basis of the described feedback inhibition of pyruvate dehydrogenase by the end-products NADH and acetyl-CoA (Garland & Randle, 1964; Bremer, 1969; Tsai *et al.*, 1973). Added to this may be a lack of saturation with the substrates NAD^+ and CoA: such an effect cannot be distinguished from the feedback inhibition in studies with intact mitochondria, owing to the near-reciprocal relation between NAD^+ and NADH and between CoA and acetyl-CoA concentrations in the mitochondrion. Interestingly, values of flux through pyruvate dehydrogenase (in State 4; Table 4*a*) are similar in the presence of palmitoylcarnitine

Table 6. *Effect of palmitoylcarnitine and acetoacetate on the [CoA]/[acetyl-CoA] and [NAD+]/[NADH] ratios of mitochondria incubated with a low concentration (60 μM) of pyruvate*

(*a*) Mitochondria were incubated as described in Table 5(*a*) for State 4 respiration, but at 0.5–1 mg of protein/ml, and were extracted and assayed for coenzyme content as indicated for Table 3. (*b*) Mitochondria were incubated as described above, but with 1 mM-EGTA replacing the Ca^{2+}–EGTA buffer. Additions to the basal medium were as noted. Data are presented as the means ± S.E.M. for six incubations, involving six mitochondrial preparations.

	Additional substrate	CoA (nmol/mg of protein)	Acetyl-CoA (nmol/mg of protein)	[CoA]/[acetyl-CoA]	NAD^+ (nmol/mg of protein)	NADH (nmol/mg of protein)	[NAD^+]/[NADH]
(*a*)	None	0.063 ± 0.010	0.52 ± 0.01	0.12	2.08 ± 0.13	0.72 ± 0.08	3.0
	Palmitoylcarnitine	0.017 ± 0.003	0.14 ± 0.01	0.12	1.72 ± 0.16	1.02 ± 0.13	1.8*
	2-Oxoglutarate	0.17 ± 0.02	0.33 ± 0.06	0.57	2.20 ± 0.03	0.59 ± 0.03	3.8
	2-Oxoglutarate + acetoacetate	0.058 ± 0.015	0.47 ± 0.03	0.13*	2.09 ± 0.08	0.94 ± 0.03	2.2*
(*b*)	None	0.48 ± 0.07	0.18 ± 0.03	3.3 ± 1.0	1.77 ± 0.08	0.90 ± 0.09	2.0 ± 0.2
	Palmitoylcarnitine	0.03 ± 0.01	0.09 ± 0.01	0.45 ± 0.22	1.41 ± 0.07	1.41 ± 0.01	1.0 ± 0.04
	2-Oxoglutarate	0.51 ± 0.06	0.16 ± 0.03	3.70 ± 0.9	2.07 ± 0.03	0.79 ± 0.08	2.8 ± 0.3
	2-Oxoglutarate + acetoacetate	0.59 ± 0.05	0.15 ± 0.01	4.10 ± 0.05	2.20 ± 0.1	0.84 ± 0.05	2.5 ± 0.2

* Indicates a significant ($P < 0.05$) effect of palmitoylcarnitine or acetoacetate.

and of acetoacetate, as are values for PDH_A content (Table 5*a*). This defines a similar degree of feedback inhibition of pyruvate dehydrogenase, operating under the prevailing conditions of the mitochondrial matrix, entirely consistent with the similar magnitudes of the [CoA]/[acetyl-CoA] and [NAD^+]/[NADH] ratios under these conditions (Table 6*a*).

In the absence of Ca^{2+} (Table 6*b*), palmitoylcarnitine oxidation induces decreases in both [CoA]/[acetyl-CoA] and [NAD^+]/[NADH] ratios. This is expected as a consequence of fatty acid oxidation. Such decreases provide a plausible mechanism for the decrease in the content of PDH_A, which is a consequence of palmitoylcarnitine oxidation under these conditions (Table 5*b*). However, they would also be expected to give an increased degree of feedback inhibition of PDH_A, as obtained by comparing flux (Table 4*b*) with PDH_A content (Table 5*b*).

This is not seen, and this is a puzzle. Comparison of the [CoA]/[acetyl-CoA] ratio obtained in the presence of the pyruvate-generating system alone in Table 6(*a*) with that of Table 6(*b*) provides a graphic illustration of the effect of extra-mitochondrial Ca^{2+}, at pCa 6.48. This raises the PDH_A content from 33 to 91.4 nmol/min per mg (Table 5): with flux ultimately restrained by the respiratory chain in State 4, the raised PDH_A content then requires an increased degree of end-product inhibition (by acetyl-CoA) so that NADH generation and oxidation remain matched. This is what is seen.

The lack of effect of acetoacetate on these effector ratios in the absence of Ca^{2+} (Table 6*b*) tends to reinforce the idea that it is not being oxidized, owing to poor 2-oxoglutarate dehydrogenase activity in the absence of Ca^{2+}. Thus the question of whether acetoacetate oxidation, were it adequately activated by succinyl-CoA, would inhibit flux through pyruvate dehydrogenase in the absence of Ca^{2+} remains open.

Discussion

The predominance of fatty acid and ketone oxidation over carbohydrate oxidation which has been established in cardiac muscle (see the introduction for references) has not been so clearly shown for skeletal muscle, and this has been reviewed by Berger *et al.* (1976), Rennie & Holloszy (1977) and Ruderman *et al.* (1979). There is good evidence that starvation and diabetes are both associated with a decreased PDH_A content of skeletal muscle (Hennig *et al.*, 1975; Hagg *et al.*, 1976) and that this effect can be mimicked in the perfused rat hindquarter by perfusion with a medium containing acetoacetate (Hagg *et al.*, 1976). This effect is seen in resting muscle: when the muscle is stimulated electrically, the PDH_A content is raised (Hennig *et al.*, 1975; Hagg *et al.*, 1976), but responsiveness to acetoacetate is lost (Hagg *et al.*, 1976). However, flux through pyruvate dehydrogenase, as measured by lactate oxidation, is diminished by the presence of acetoacetate in both resting and stimulated muscle (Berger *et al.*, 1976). Presumably, increased feedback inhibition can be invoked as the mechanism of decreased flux in the absence of changes in PDH_A content in the stimulated muscle. Indeed, an increased [acetyl-CoA]/[CoA] ratio has been determined in muscle, during perfusion of the hindquarter with acetoacetate (Berger *et al.*, 1976). However, the use of the perfused hindquarter, while allowing the maintenance of the muscle mass in a near-physiological condition (see Ruderman *et al.*, 1971; Rennie & Holloszy, 1977), precludes the

measurement of [CoA]/[acetyl-CoA], [NAD^+]/[NADH] and [ATP]/[ADP] ratios in the mitochondrial matrix, which would be appropriate for a discussion of pyruvate dehydrogenase interconversion. For this reason, we carried out experiments with skeletal-muscle mitochondria from muscles of the hind-limb, and studied both PDH_A content and the contents of putative effectors in response to acetoacetate oxidation. Different degrees of mechanical work were mimicked by the graded stimulation of respiration by ADP, produced by the hexokinase reaction and by the varying extra-mitochondrial free Ca^{2+} concentration. Finally, the response of pyruvate dehydrogenase to fatty acid oxidation was tested with palmitoylcarnitine, in analogy to experiments previously carried out with cardiac mitochondria in this laboratory (Hansford, 1977; Hansford & Cohen, 1978) and elsewhere (Kerbey *et al.*, 1977). This was done despite the background information that the presence of palmitate has no effect on lactate oxidation by the isolated rat soleus muscle (Pearce & Connett, 1980). In these previous experiments the muscle was unstimulated, and it appeared possible that different results might be obtained with mitochondria incubated in the presence of ADP and Ca^{2+}.

It was found that palmitoylcarnitine oxidation did inhibit flux through pyruvate dehydrogenase, when mitochondria were exposed to low, plausibly physiological, concentrations of pyruvate (Table 4). However, the effect was far less dramatic than that seen with heart mitochondria (Hansford, 1977; Hansford & Cohen, 1978). This result then differs from the finding of a lack of effect of long-chain fatty acids seen with the perfused hindquarter (Berger *et al.*, 1976) or the isolated soleus muscle (Pearce & Connett, 1980). However, provision of palmitoylcarnitine to the mitochondria is optimal in the mitochondrial studies, whereas in the intact tissue fatty acid oxidation could be limited by permeation of the plasma membrane or at the level of fatty acid activation in the cytosol (Neely *et al.*, 1972). Thus there is not necessarily a conflict here.

The availability of Ca^{2+} is an over-riding factor in determining the content of PDH_A (Table 2) and susceptibility to effects of fatty acid oxidation (Table 5). It is noteworthy that an incubation medium of pCa 6.48 (i.e. containing $0.33\,\mu$M free Ca^{2+}) can give such a dramatically different effect from a medium containing EGTA, especially since the 10 mM-NaCl and 1 mM-$MgCl_2$ present will tend to minimize the extent of the Ca^{2+} gradient across the mitochondrial inner membrane (Denton *et al.*, 1980; Hansford, 1981; see Nicholls & Åkerman, 1982, for a review). This emphasizes the ability of skeletal-muscle mitochondria to withdraw Ca^{2+} from media of very low ambient Ca^{2+} concentration. The effect of Ca^{2+} in these experiments is quite separate from indirect effects of Ca^{2+} which are obtained at pCa values of 6 and less, and which reflect a partial collapse of the mitochondrial membrane potential, with a consequent activation of respiration and decrease of [ATP]/[ADP] and [NADH]/[NAD^+] ratios. If experiments are performed with Ca^{2+} buffers of pCa 6 and less, a massive accumulation of Ca^{2+} and deterioration of mitochondrial oxidative function may occur. Under these conditions, PDH_A values tend to decrease, as seen at the lowest pCa value in Fig. 1. In the present paper, the value of pCa used for most experiments was 6.48: at this value, State-4 respiration, a sensitive indicator of the mitochondrial proton electrochemical gradient, is not elevated above values obtained in the presence of EGTA. More discussion on this matter, together with experimental data, may be found in Hansford (1981).

Flux through pyruvate dehydrogenase was also found to be inhibited on the addition of acetoacetate (Table 4), consistent with results obtained for the perfused hindquarter. The inhibition was limited to those conditions under which acetoacetate was effectively oxidized, i.e. in the presence of 0.5 mM-2-oxoglutarate and Ca^{2+}.

When PDH_A content was measured, rather than flux, an effect of fatty acid oxidation was only noted in the absence of Ca^{2+} (Table 5): the analogous experiments with acetoacetate were marred by inactive oxidation of the ketone under these conditions (see above). This pronounced effect of Ca^{2+} in poising the interconversion system in favour of PDH_A, which was mentioned above, many underlie the lack of effect of acetoacetate oxidation on PDH_A content of the contracting muscles of the hind-limb (Hagg *et al.*, 1976). Frequent electrical stimulation may reasonably be expected to raise the time-averaged cytosolic free Ca^{2+} concentration, with the same consequences as when isolated mitochondria are incubated at pCa 6.48, rather than pCa $>$ 8 (i.e. in the presence of EGTA); see Table 5. In this vein, it seems that the most relevant incubation conditions in this study are 'State 4 plus EGTA' and '50% or 80% State 3 plus Ca^{2+}-EGTA, pCa 6.48', i.e. 'minus ADP minus Ca^{2+}' and 'plus (limiting) ADP plus Ca^{2+}'. These states may approach those of rest and contraction, respectively, for the intact muscle. The inclusion of Ca^{2+} in this modified view of respiratory control is important when the substrate oxidation involves a Ca^{2+}-sensitive dehydrogenase, as it does for pyruvate. If data are abstracted from Table 4 corresponding to these conditions, the 'rest-to-activity' transition would be associated with an increase in pyruvate dehydrogenase flux from 21 to 72 nmol/min per mg of protein in the absence of fatty acid and from 18 to 39 nmol/min per mg of protein in the presence of palmitoylcarnitine.

The measurements of [CoA]/[acetyl-CoA] and

[NAD+]/[NADH] ratios can be interpreted at two different levels. On the first, they provide part of the mechanism underlying changes in PDH_A content. Thus the decreased PDH_A content seen to accompany the oxidation of palmitoylcarnitine in the absence of pyruvate (Table 2) is associated with a large decrease in the [CoA]/[acetyl-CoA] ratio, but with no change in [NAD+]/[NADH] ratio (Table 3). The former change, then, may reasonably provide the mechanism of changed PDH_A content, via alterations in kinase activity (Pettit *et al.*, 1975; Cooper *et al.*, 1975; Kerbey *et al.*, 1976). On the second level, a comparison of flux through pyruvate dehydrogenase (Table 4) with PDH_A content under the same conditions (Table 5) allows an estimate of the extent to which the enzyme is not functioning under $V_{max.}$ conditions in its intramitochondrial milieu. Inspection of the [NAD+]/[NADH] and [CoA]/[acetyl-CoA] ratios (Table 6) then allows a description of the way in which the concentrations of reactants and products deviate from the optimal (i.e. $V_{max.}$ conditions). For instance, the addition of acetoacetate in State 4 in the presence of Ca^{2+} decreases flux through pyruvate dehydrogenase by 36% (Table 4), without a corresponding decrease in PDH_A content (Table 5). This implies increased feedback inhibition, which can be attributed in this case to a decrease in both [CoA]/[acetyl-CoA] and [NAD+]/[NADH] ratios (Table 6). Increased whole-tissue acetyl-CoA content has been described as a function of acetoacetate oxidation (Berger *et al.*, 1976): in this complementary study, the use of isolated mitochondria allows the additional description of redox changes of the nicotinamide nucleotide of the mitochondrial compartment.

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References

Azzone, G. F. & Carafoli, E. (1960) *Exp. Cell Res.* **21**, 447–467

Batenburg, J. J. & Olson, M. S. (1976) *J. Biol. Chem.* **251**, 1364–1370

Berger, M., Hagg, S. A., Goodman, M. N. & Ruderman, N. B. (1976) *Biochem. J.* **158**, 191–202

Bremer, J. (1969) *Eur. J. Biochem.* **8**, 535–540

Cate, R. L. & Roche, T. E. (1978) *J. Biol. Chem.* **253**, 496–503

Chance, B. & Williams, G. R. (1956) *Adv. Enzymol.* **17**, 65–134

Chappell, J. B. & Perry, S. V. (1954) *Nature (London)* **173**, 1094–1095

Chen, J. C., Warshaw, J. B. & Sanadi, D. R. (1972) *J. Cell. Physiol.* **80**, 141–148

Cooper, R. H., Randle, P. J. & Denton, R. M. (1975) *Nature (London)* **257**, 808–809

Davis-van Thienen, W. & Davis, E. J. (1981) *J. Biol. Chem.* **256**, 8371–8378

Dennis, S. C., Padma, A., Debuysere, M. S. & Olson, M. S. (1979) *J. Biol. Chem.* **254**, 1252–1258

Denton, R. M., Randle, P. J. & Martin, B. R. (1972) *Biochem. J.* **128**, 161–163

Denton, R. M., McCormack, J. G. & Edgell, N. J. (1980) *Biochem. J.* **190**, 107–117

Evans, J. R., Opie, L. H. & Renold, A. E. (1963) *Am. J. Physiol.* **205**, 971–976

Garland, P. B. & Randle, P. J. (1964) *Biochem. J.* **91**, 6c–7c

Garland, P. B., Newsholme, E. A. & Randle, P. J. (1964*a*) *Biochem. J.* **93**, 409–427

Garland, P. B., Newsholme, E. A. & Randle, P. J. (1964*b*) *Biochem. J.* **93**, 665–678

Hagg, S. A., Taylor, S. I. & Ruderman, N. B. (1976) *Biochem. J.* **158**, 203–210

Hansford, R. G. (1976) *J. Biol. Chem.* **251**, 5483–5489

Hansford, R. G. (1977) *J. Biol. Chem.* **252**, 1552–1560

Hansford, R. G. (1978) *Biochem. J.* **170**, 285–295

Hansford, R. G. (1981) *Biochem. J.* **194**, 721–732

Hansford, R. G. (1983) *Biochim. Biophys. Acta* **726**, 41–80

Hansford, R. G. & Castro, F. (1981) *Biochem. J.* **198**, 525–533

Hansford, R. G. & Cohen, L. (1978) *Arch. Biochem. Biophys.* **191**, 65–81

Hennig, G., Löffler, G. & Wieland, O. H. (1975) *FEBS Lett.* **59**, 142–145

Hucho, F., Randall, D. D., Roche, T. E., Burgett, M. W., Pelley, J. W. & Reed, L. J. (1972) *Arch. Biochem. Biophys.* **151**, 328–340

Hutson, N. J. & Randle, P. J. (1978) *FEBS Lett.* **92**, 73–76

Kerbey, A. L. & Randle, P. J. (1981) *FEBS Lett.* **127**, 188–192

Kerbey, A. L., Randle, P. J., Cooper, R. H., Whitehouse, S., Pask, H. T. & Denton, R. M. (1976) *Biochem. J.* **154**, 327–348

Kerbey, A. L., Radcliffe, P. M. & Randle, P. J. (1977) *Biochem. J.* **164**, 509–519

Linn, T. C., Pettit, F. H. & Reed, L. J. (1969*a*) *Proc. Natl. Acad. Sci. U.S.A.* **62**, 234–241

Linn, T. C., Pettit, F. H., Hucho, F. & Reed, L. J. (1969*b*) *Proc. Natl. Acad. Sci. U.S.A.* **64**, 227–234

McCormack, J. G. & Denton, R. M. (1979) *Biochem. J.* **180**, 533–544

Mickelson, J. R., Greaser, M. L. & Marsh, B. B. (1980) *Anal. Biochem.* **109**, 255–260

Neely, J. R. & Morgan, H. E. (1974) *Annu. Rev. Physiol.* **36**, 413–459

Neely, J. R., Bowman, R. H. & Morgan, H. E. (1969) *Am. J. Physiol.* **216**, 804–811

Neely, J. R., Rovetto, M. J. & Oram, J. R. (1972) *Proc. Cardiovasc. Dis.* **15**, 289–329

Newsholme, E. A. & Randle, P. J. (1964) *Biochem. J.* **93**, 641–651

Nicholls, D. & Åkerman, K. (1982) *Biochim. Biophys. Acta* **683**, 57–88

Olson, M. S., Dennis, S. C., DeBuysere, M. S. & Padma, A. (1978) *J. Biol. Chem.* **253**, 7369–7375

Pande, S. V. & Blanchaer, M. C. (1971) *Am. J. Physiol.* **220**, 549–553

Pearce, F. J. & Connett, R. J. (1980) *Am. J. Physiol.* **238**, C149–C159

Pettit, F. H., Roche, T. E. & Reed, L. J. (1972) *Biochem. Biophys. Res. Commun.* **49**, 563–571

Pettit, F. H., Pelley, J. W. & Reed, L. J. (1975) *Biochem. Biophys. Res. Commun.* **65**, 575–582

Portzehl, H., Caldwell, P. C. & Rüegg, J. C. (1964) *Biochim. Biophys. Acta* **79**, 581–591

Randle, P. J., Garland, P. B., Hales, C. N. & Newsholme, E. A. (1963) *Lancet* **i**, 785–789

Randle, P. J., Newsholme, E. A. & Garland, P. B. (1964) *Biochem. J.* **93**, 652–665

Randle, P. J., Garland, P. B., Hales, C. N., Newsholme, E., Denton, R. M. & Pogson, C. I. (1966) *Recent Prog. Horm. Res.* **22**, 1–44

Randle, P. J., England, P. J. & Denton, R. M. (1970) *Biochem. J.* **117**, 677–695

Randle, P. J., Denton, R. M., Pask, H. T. & Severson, D. (1974) *Biochem. Soc. Symp.* **39**, 75–87

Rennie, M. J. & Holloszy, J. O. (1977) *Biochem. J.* **168**, 161–170

Ruderman, N. B., Houghton, C. R. S. & Hems, R. (1971) *Biochem. J.* **124**, 639–651

Ruderman, N. B., Goodman, M. N., Conover, C. A. & Berger, M. (1979) *Diabetes* **28**, *Suppl.* **1**, 13–17

Severson, D. L., Denton, R. M., Pask, H. T. & Randle, P. J. (1974) *Biochem. J.* **140**, 225–237

Tsai, C. S., Burgett, M. W. & Reed, L. J. (1973) *J. Biol. Chem.* **248**, 8348–8352

Van Hinsbergh, V. W. M., Veerkamp, J. H. & Van Moerkerk, H. T. B. (1978) *Arch. Biochem. Biophys.* **190**, 767–771

Whitehouse, S., Cooper, R. H. & Randle, P. J. (1974) *Biochem. J.* **141**, 761–774

Wieland, O., Funcke, H. V. & Löffler, G. (1971) *FEBS Lett.* **15**, 295–298