An improved assay for pyruvate dehydrogenase in liver and heart

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A radiochemical assay was developed to measure pyruvate dehydrogenase complex (PDC) activity in liver and heart without interference by branched-chain 2-oxo acid dehydrogenase (BCODH). Decarboxylation of pyruvate by BCODH was eliminated by using low pyruvate concentration (0.5 mM), a preferred substrate for BCODH (3-methyl-2-oxopentanoate) that is not used by PDC, and a competitive inhibitor of BCODH, dichloroacetate. This method was validated by assaying a combination of both purified enzymes and tissue homogenates with known amounts of added BCODH. The actual percentage of active PDC decreased after 48 h starvation from 13.6 to 3.1 in liver and from 77.1 to 9.0 in heart. Total PDC activity (munits of PDC/units of citrate synthase) in starved rats was increased by 34 % in liver and decreased by 23 % in heart. Total PDC activity (munits/g wet wt.) in fed- and starved-rat liver was 0.8 and 1.3, and in heart was 6.6 and 5.8, respectively.

INTRODUCTION

Pyruvate dehydrogenase complex (PDC) (EC 1.2.4.1 + EC 2.3.1.12 + EC 1.8.1.4) and branched-chain 2-oxo acid dehydrogenase (BCODH) (EC 1.2.4.4 + no EC number for dihydrolipoamide acyltransferase + EC 1.8.1.4) are intramitochondrial multienzyme complexes composed of three enzymes together with a kinase (no EC number) that phosphorylates and inactivates the complex, and a phosphatase (no EC number) that dephosphorylates and activates the complex [1–4]. Both complexes oxidatively decarboxylate 2-oxo acids, producing CO₂, acyl-CoA and NADH. They show some overlap in their 2-oxo acid specificities with the most notable being pyruvate and 2-oxobutyrate [3,5–7]. Use of 2-oxobutyrate by both complexes, particularly by BCODH, is physiologically important in threonine and methionine catabolism [5,7]. Whether or not pyruvate is a physiological substrate for BCODH is not clear, because its K_m is very high (820 μ M) compared with that for PDC of 50 μ M [5,6,8]. However, the use of pyruvate decarboxylation in tissue extracts as a measure of PDC may lead to overestimation of PDC activity [5,8] and to erroneous conclusions regarding the effects of the branched-chain 2-oxo acids on pyruvate use by PDC as a possible mechanism behind abnormalities associated with maple-syrup-urine disease (see [5]). The extent of these problems depends on the assay pyruvate concentration, because of the different K_m values of both complexes, on the tissue, because of the relative activities of both complexes, and on the animal's physiological condition, because of the possible different effects on the activities of both complexes.

PDC and BCODH activities measured with pyruvate are indistinguishable by spectrophotometric or radiochemical techniques, because all substrates and end products are the same. True PDC activity in tissue extracts can only be measured if BCODH activity is eliminated [5,8]. In tissues such as liver, where the ratio of total BCODH to total PDC activity is about 0.65 and where BCODH is > 85 % active and PDC is < 15 % active [1,5,9–11], the possibility of a significant overestimation of PDC activity is likely, particularly if high pyruvate concentration (> 0.5 mM) is used [5,8]. One method to eliminate the contribution of BCODH to PDC overestimation used inhibitory antibodies to BCODH [5,8]. This technique, however, has limited application because isolation of BCODH and subsequent gen-

eration of antibody is time-consuming, the antibodies are not readily available, and because of the possibility that antibodies against BCODH may cross-react with PDC, particularly involving the dihydrolipoamide dehydrogenase (EC 1.8.1.4) component of both complexes.

The purpose of this study was to develop an assay using readily available reagents to measure actual PDC activity in tissue extracts that eliminates interference by BCODH activity, and to use this assay to show the effect of 48 h starvation on the expressed and total PDC activity in rat liver and heart.

EXPERIMENTAL

Materials

Reagents were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. [1-¹⁴C]Pyruvate (6.00 mCi/mmol) was purchased from DuPont NEN Products. Bovine heart PDC (17.9 units/mg of protein) was purified [12] with an additional step of chromatography on hydroxyapatite [5]. Rabbit liver BCODH (7.1 units/mg of protein) [2,13], goat antibody (IgG) to BCODH [5] and broad-specificity protein phosphatase (1.5 mg of BCODH dephosphorylated/min per ml), which activates both PDC and BCODH [10,13–15], were obtained as described. All enzymes were stored at –70 °C in storage buffer containing 15 mM-Hepes, 0.05 mM-EDTA, 0.05 mM-thiamin pyrophosphate, 5 mM-dithiothreitol, 0.015 % (v/v) Triton X-100 and 15 % (v/v) glycerol, pH 7.5. Male Wistar rats (175–200 g) were purchased from Harlan Sprague-Dawley Corp. Rats were allowed water and food (Teklad Premium Lab. Diet, Rodent Blox 8640; $\geq$ 22 % crude protein) *ad libitum*. Starved rats were given water *ad libitum*, but no food for 48 h before death.

Enzyme assays

Radiochemical and spectrophotometric assays, with total volumes of 0.2 and 1.0 ml respectively, contained 0.05 mM-[1-¹⁴C]pyruvate (1200 c.p.m./nmol), 50 mM-Hepes/K⁺, 6.5 mM-NAD⁺, 0.5 mM-CoA, 0.4 mM-thiamin pyrophosphate, 0.3–5 units of lipoamide dehydrogenase, 2.0 mM-dithiothreitol, 2 mM-MgCl₂, pH 7.5 at 30 °C, and either isolated enzymes or tissue extracts (see below). When present, goat anti-BCODH IgG, 3-methyl-2-oxopentanoate and dichloroacetate (DCA) were at final concentrations of 70 μ g/ μ g of PDC or BCODH, 3 mM and

Abbreviations used: PDC, pyruvate dehydrogenase complex; BCODH, branched-chain 2-oxo acid dehydrogenase; DCA, dichloroacetate.

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3 mM respectively. The radiochemical pyruvate decarboxylation assay [5,10,14] and the spectrophotometric NADH-generation assay [12] were as described. Citrate synthase was assayed as described in [16]. All assays were linear with time and enzyme amount (see results). One unit is 1 $\mu\text{mol}/\text{min}$ at 30 °C.

Tissue assays

Rats were quickly decapitated and the hearts and livers were immediately removed, freeze-clamped in liquid N_2 and stored at -70 °C. Frozen tissues were placed in 20 vol. (v/w) of ice-cold homogenizing buffer [50 mM-Hepes/K, 5 mM-EDTA, 2 mM-dithiothreitol, 1% (v/v) Triton X-100, 0.2 mM-thiamin pyrophosphate, 50 μg of trypsin inhibitor/ml, 0.5 μM -leupeptin, 0.5 μM -pepstatin A, 0.1 mM-tosyl-L-lysylchloromethane, 1 μg of aprotinin/ml, 1 μg of chymostatin/ml and 1% (v/v) bovine serum, pH 7.5 at 20 °C] and homogenized with a Polytron homogenizer at maximum speed for 30 s, frozen in liquid N_2 , thawed, and homogenized again, first with a motor-driven Potter–Elvehjem homogenizer and then with a Polytron at full speed for 10 s. Homogenates were centrifuged for 8 min at 14000 rev./min in a refrigerated Eppendorf 5415 C centrifuge and were always maintained on ice or at ice temperature unless indicated.

After centrifugation, supernatants were divided to measure the active or expressed form of PDC and the total PDC activity [10,14]. One part was immediately adjusted to 25 mM-KF and 25 mM-potassium phosphate with ice-cold 0.5 M solution and was used to measure the active form of PDC. Another portion of the homogenate (11.5 μl) was diluted with 138.5 μl of ice-cold homogenizing buffer adjusted to 5.5 mM- MgCl_2 , and 15 μl of broad-specificity phosphatase was added. After incubation at 30 °C for 15 min, the mixture was adjusted to 25 mM-potassium phosphate and 25 mM-KF as above to inhibit the phosphatase and to make the supernatant equal in ionic composition to the other portion of the homogenate. The time course of phosphatase activation of PDC activity or pyruvate decarboxylation was complete under these conditions by 10 min. The phosphatase activates both PDC and BCODH [5,10,13–15].

Data analysis

A two-tailed paired or unpaired Student's *t* test was used to compare groups. Values were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Decarboxylation of [$1\text{-}^{14}\text{C}$]pyruvate by isolated PDC was linear ($r = 0.998$) over time or enzyme amount up to the generation of 20 nmol of CO_2 (results not shown). The assay's design was based on the use of a much better 2-oxo acid substrate for BCODH than pyruvate and the use of a competitive inhibitor of BCODH's use of 2-oxo acids. 3-Methyl-2-oxopentanoate was selected as an alternative substrate for BCODH because its K_m value (8–14 μM) [2,17] is about 55 times lower than for pyruvate (820 μM) [5]. Additionally, it would not be used as quickly as other branched-chain 2-oxo acids, because it has the lowest V_{max} [2,17], and it does not inhibit PDC at relatively high concentration (5 mM). DCA was used to inhibit BCODH activity [18] because of its lack of effect on PDC activity with the stated conditions and because of its ready availability. The combination of 3 mM-3-methyl-2-oxopentanoate and DCA was chosen on the basis of combinations of the two reagents in the ranges 0–15 mM-DCA and 0–16 mM-3-methyl-2-oxopentanoate. DCA plus 3-methyl-2-oxopentanoate, each at 3 mM, did not inhibit PDC activity ($2 \pm 1\%$ inhibition, $n = 32$; Table 1). Lower concentrations of the two compounds did not completely inhibit BCODH, but higher concentrations inhibited PDC activity.

The spectrophotometric (NADH generation) and radiochemical (CO_2 generation) assays were in good absolute agreement for PDC, BCODH and the combination of PDC and BCODH activities (Table 1). DCA and 3-methyl-2-oxopentanoate did not inhibit either assay of PDC, and completely eliminated pyruvate decarboxylation by BCODH (Table 1). Spectrophotometric assay of BCODH activity with pyruvate could not be done in the presence of 3-methyl-2-oxopentanoate, because it is oxidatively decarboxylated by BCODH to generate NADH. When both PDC and BCODH are present, as is true for tissue or mitochondrial extracts, the presence of 3-methyl-2-oxopentanoate and DCA completely eliminated pyruvate decarboxylation by BCODH (Table 1 and Fig. 1a). Inhibition of BCODH decarboxylation of pyruvate by 3-methyl-2-oxopentanoate and DCA is as effective as the use of inhibitory antibodies to BCODH (Table 1), another procedure that eliminates BCODH activity [5,8].

The effectiveness of 3-methyl-2-oxopentanoate and DCA in eliminating BCODH activity towards pyruvate was demonstrated with both isolated enzymes (Fig. 1a) and with tissue extracts supplemented with BCODH (Fig. 1b, heart; Fig. 1c, liver). Interference by up to 3 times more BCODH activity than PDC activity (based on use of 0.5 mM-pyruvate) could be effectively eliminated from pyruvate decarboxylation (Fig. 1a). This corresponds to about 40 times more BCODH activity, based on its V_{max} with 2-methyl-2-oxobutyrate [5], than PDC activity measured with 0.5 mM-pyruvate. There was no difference in pyruvate decarboxylation between pyruvate alone and pyruvate with 3-methyl-2-oxopentanoate plus DCA in heart extract without added BCODH, because BCODH in fed rat heart is only 10–45% active [9,19,20], whereas PDC is about 60–80% active [21–23]; Table 2) and total PDC activity is about 15-fold greater than BCODH activity [5]. In contrast, there was a difference between pyruvate alone and pyruvate with 3-methyl-2-oxopentanoate plus DCA in liver extracts without added BCODH, because total liver BCODH is greater than PDC [5], with the percentage of active form of liver BCODH $> 85\%$ [7,24–28] and that for PDC $< 15\%$ [8,11,29–32]; Table 2).

Significant overestimation of hepatic expressed and total PDC activity existed for both fed and starved rats (Table 2). The percentage of active hepatic PDC was also overestimated in fasted rats. Overestimation of heart PDC activity occurred in expressed activity in starved rats, and in total activity in both fed and starved rats (Table 2). The degree of overestimation will depend on the assay pyruvate concentration, the relative tissue activities of PDC and BCODH, and the animals' physiological state (i.e. fed or starved). The assay pyruvate concentration used in the present study was 10 times the K_m for PDC, but only about 0.6 times its K_m with BCODH. With increasing pyruvate concentration, the increased PDC activity would be minor relative to the increased contribution of BCODH to pyruvate decarboxylation. For example, PDC activity would increase, assuming that NAD^+ and CoA remained constant, by about 4% with 1 mM-pyruvate relative to 0.5 mM, whereas pyruvate decarboxylation by BCODH would increase by about 17% [5]. The degree of overestimation also would depend on the relative amounts of active PDC and BCODH in a particular tissue. The ratio of total PDC to BCODH activity is about 0.65 in liver and about 15 in heart [5]. Thus, given the same assay pyruvate concentration, the amount of overestimation will be greater in liver than in heart (Table 2). Finally, the physiological state of the animal can independently affect the total amount and the expressed activity of either complex, and, consequently, the degree of overestimation (Table 2). For example, BCODH in fed (about 20% protein)-rat liver is nearly completely active, and starvation has little effect on the activity state [7,24–28], whereas

Table 1. Activities of PDC and BCODH assayed with pyruvate, or pyruvate plus 3-methyl-2-oxopentanoate and DCA (PMOD), or pyruvate plus antibodies (IgG) to BCODH

Activities (nmol/min) are means ± S.E.M. for 3–7 independent observations. See the Experimental section for details. ‘Expected’ values are based on spectrophotometric assay with PDC (0.13 µg/assay), BCODH (0.98 µg/assay) or a combination of PDC (0.06 µg/assay) and BCODH (0.49 µg/assay). There were no significant differences (*P* > 0.05) between spectrophotometric and radiochemical assays, between expected and observed values, nor between the observed/expected ratio and 100 %.

Treatment	Activity			
	Assay ...	Spectrophotometric		[1- ¹⁴ C]Pyruvate decarboxylation
		Observed	Expected	Observed expected
PDC		0.88 ± 0.09	0.88	0.86 ± 0.05 98 %
PDC + PMOD		0.86 ± 0.06	0.88	0.86 ± 0.06 98 %
PDC + IgG		0.87 ± 0.04	0.88	0.74 ± 0.08 84 %
BCODH		0.43 ± 0.02	0.43	0.46 ± 0.02 107 %
BCODH + PMOD		—*	0.00	0.01 ± 0.01 —
BCODH + IgG		0	0.00	0.00 ± 0.00 —
PDC + BCODH		1.25 ± 0.20	1.31	1.41 ± 0.12 108 %
PDC + BCODH + PMOD		—*	0.88	0.77 ± 0.04 88 %
PDC + BCODH + IgG		0.79 ± 0.14	0.88	0.68 ± 0.06 77 %

* Spectrophotometric assay could not be done because BCODH uses both pyruvate and 3-methyl-2-oxopentanoate.

Fig. 1. PDC activity in the presence of BCODH

All reactions were initiated with either 0.5 mM-pyruvate (○) or 0.5 mM-pyruvate, 3 mM-DCA and 3 mM-3-methyl-2-oxopentanoate (●). (a) Reaction combined 0.03 µg of PDC (17.9 units/mg) and increasing amounts (0, 0.25, 0.49, 0.98, 1.46 µg) of BCODH (7.1 units/mg), based on 0.5 mM-pyruvate. Homogenates of fed rat (b) heart and (c) liver (see the Experimental section) were assayed as given above with increasing amounts of BCODH (A = no BCODH; B = 0.43 µg of BCODH; C = 0.86 µg of BCODH; D = 1.29 µg of BCODH). Vertical lines represent 1 S.E.M.

PDC is about 15 % active in fed rat liver, and starvation decreases the percentage of active form to < 5 % [8,11,23,29,31,32]; Table 2). Consequently, it is difficult to assess the *a priori* contribution

of BCODH to overestimation of PDC in any given tissue from animals of different physiological states. It is therefore imperative to eliminate BCODH’s contribution to pyruvate decarboxylation if effects of physiological conditions on PDC activity are to be elucidated.

Starvation decreased the percentage of active PDC in liver from about 14 to 3 % and in heart from about 77 to 9 % (Table 2). Several reports [8,11,23,29–32] on the effect of starvation also show a decreased percentage of active PDC in liver and heart, with heart showing the largest change. The decreased percentage of active PDC or increased phosphorylation state with starvation likely occurs because PDC kinase is activated by increased intramitochondrial NADH/NAD⁺ and acyl-CoA/CoA pools [4,33,34] generated from increased fatty acid oxidation. Other mechanisms that may also contribute to increased PDC phosphorylation or inactivation with starvation include increased amounts of PDC kinase and of kinase activator protein [35,36].

The qualitative results in the present paper agree well with previous studies, although there are some distinctive quantitative differences that possibly result from using a better assay. Total hepatic PDC activity per g wet wt. increased by about 62 % with starvation. When normalized to citrate synthase, the total PDC was increased by 34 %, from 71 to 96 m-units of PDC/unit of citrate synthase. However, the total PDC activity/liver does not change with starvation, because liver weight is about 40 % less in the starved animal [37]. The change in total PDC activity per g wet wt. and per unit of citrate synthase with starvation was possibly not seen in other studies [11,29,31,32,38] because of overestimation of PDC activity by BCODH in both fed and starved animals. Additionally, lower values of citrate synthase obtained in the other studies probably indicate incomplete tissue homogenization. Citrate synthase has been used as a marker for the degree of solubilization of mitochondrial enzymes (see [23]). However, it may not be an ideal marker for large multienzyme complexes, because of its possible association with different intramitochondrial components and because of its vastly different size [27]. If starvation does not affect expression of citrate synthase or mitochondrial number per cell, then citrate synthase activity and cell number per g wet wt. of liver should increase

Table 2. PDC expressed and total activities and citrate synthase (CS) activity in fed and 48 h-starved rat liver and heart measured with pyruvate and pyruvate + 3-methyl-2-oxopentanoate + DCA (PMOD)

Activities (means $\pm$ S.E.M.; $n = 6$ for liver and $n = 5$ for heart) are given as munits/g wet wt. for PDC and as units/g wet wt. for CS. Statistical significance ($P < 0.05$) between conditions, based on a paired Student's t test (pyruvate versus PMOD), is indicated by 'a', and based on non-paired Student's t test (fed versus starved) is indicated by 'b'. 'Overestimation' is activity measured with only pyruvate divided by the activity measured with PMOD.

	Liver		Heart	
	Fed	Starved	Fed	Starved
PDC				
Expressed activity				
Pyruvate	143 $\pm$ 35	78 $\pm$ 8	6560 $\pm$ 817	800 $\pm$ 141 ^b
PMOD	110 $\pm$ 30	40 $\pm$ 10 ^b	5560 $\pm$ 676	550 $\pm$ 92 ^b
Overestimation	27 % ^a	100 % ^a	18 %	46 % ^a
Total activity				
Pyruvate	969 $\pm$ 104	1428 $\pm$ 87 ^b	8440 $\pm$ 1016	7960 $\pm$ 349
PMOD	800 $\pm$ 66	1300 $\pm$ 79 ^b	7320 $\pm$ 945	6160 $\pm$ 227
Overestimation	21 % ^a	10 % ^a	15 % ^a	29 % ^a
100 $\times$ Expressed/total (% active)				
Pyruvate	14.2 $\pm$ 2.6	5.6 $\pm$ 0.7 ^b	79.4 $\pm$ 8.3	10.1 $\pm$ 1.8 ^b
PMOD	13.6 $\pm$ 4.3	3.1 $\pm$ 1.4 ^b	77.1 $\pm$ 6.0	9.0 $\pm$ 1.5 ^b
Overestimation	4 %	81 % ^a	1.0 %	1.1 %
CS	11.4 $\pm$ 0.49	13.6 $\pm$ 0.44 ^b	119.1 $\pm$ 15.0	129.5 $\pm$ 4.4
Total PDC/CS (m-units/unit)	71.0 $\pm$ 7.3	95.5 $\pm$ 5.3 ^b	62.1 $\pm$ 7.8	47.6 $\pm$ 1.5 ^b

with starvation because of the absence of glycogen and its associated water. The present work shows citrate synthase activity per g wet wt. increased by about 20 % with starvation (Table 2). Several studies [27,39], including the present one (Table 2), have measured > 11 units/g wet wt., whereas most studies (see above) of fed- and starved-animal PDC activity reported 8–9.5 units of citrate synthase/g wet wt. and no difference in citrate synthase with starvation. Thus, differences in PDC activity seen in the present study relative to other reports [11,29,31,32,38] may reflect differences in PDC assay and tissue homogenization.

The percentage of active heart PDC varies considerably in the literature, from 60–75 % [22,23; Table 2] to 15–30 % [29,31, 32,40] in the fed animal and from 7–10 % [22,23; Table 2] to 0.2–2.0 % in the starved animal [29,31,32]. The values reported in the present paper, which lie in the higher range of these values, are likely to reflect true values *in vivo* because the methods used were identical with those documented to maintain the phosphorylation state [14], the values obtained with liver by the same methods agree with previously reported values, and the percentages of active PDC in fed- and starved-rat heart agree with some of the other reported values. The reasons for the differences in percentage of active heart PDC are unclear, but may involve assay differences and dietary influences.

In contrast with liver, however, heart total PDC was decreased by about 20 % by starvation when expressed per unit of citrate synthase activity (Table 2). Even though glycogen content in heart increases with starvation [41], its low content apparently does not alter the citrate synthase activity per g wet wt. Since heart weight also declines by about 10 % with starvation [23], there is decreased total heart PDC regardless of how the data are expressed. Other studies that failed to show decreased total heart PDC with starvation may have obtained those results because of an overestimation of PDC or decreased extraction of PDC, based on generally lower recovery of citrate synthase (75–90 units/g wet wt.) compared with the present study (> 120 units/g wet wt.; Table 2).

Accurate measurement of PDC activity is critical in under-

standing the maximum capacity for a tissue's use of pyruvate and in relating activity to flux changes with different physiological states. The procedure used in this paper eliminates a major problem, decarboxylation of pyruvate by BCODH, in estimating PDC activity. Because PDC is an intramitochondrial complex, its measured activity is also dependent on the techniques used to disrupt the mitochondria within the tissue. Citrate synthase activity is probably a good measure of tissue-extraction reproducibility. Good recovery of total citrate synthase activity is also probably a good index of absolute mitochondrial disruption, and would likely be a good measure to use to normalize PDC activity in different samples with different extraction efficiencies. Obtaining a near-maximum activity (units/g wet wt.) of citrate synthase (i.e. > 11 in liver and > 120 in heart) would indicate maximum mitochondrial disruption. However, when considerably less (< 80 %) citrate synthase is extracted, its use as a normalizing parameter is less clear, because a good stoichiometry between PDC activity and citrate synthase may not exist.

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REFERENCES

- Harper, A. E., Miller, R. H. & Block, K. P. (1984) *Annu. Rev. Nutr.* **4**, 409–454
- Paxton, R. & Harris, R. A. (1982) *J. Biol. Chem.* **257**, 14433–14439
- Yeaman, S. J. (1986) *Trends Biochem. Sci.* **11**, 293–296
- Reed, L. J. & Yeaman, S. J. (1987) *Enzymes* 3rd Ed. **18**, 77–95
- Paxton, R., Scislowski, P. W. D., Davis, E. J. & Harris, R. A. (1986) *Biochem. J.* **234**, 295–303
- Pettit, F. H., Yeaman, S. J. & Reed, L. J. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4881–4885
- Jones, S. M. A. & Yeaman, S. J. (1986) *Biochem. J.* **237**, 621–623

8. Goodwin, G. W., Paxton, R., Gillim, S. E. & Harris, R. A. (1986) *Biochem. J.* **236**, 111–114
9. Gillim, S. E., Paxton, R., Cook, G. A. & Harris, R. A. (1983) *Biochem. Biophys. Res. Commun.* **111**, 74–81
10. Paxton, R., Harris, R. A., Sener, A. & Malaisse, M. J. (1988) *Horm. Metab. Res.* **20**, 317–322
11. Wieland, O. H., Patzelt, C. & Löffler, G. (1972) *Eur. J. Biochem.* **26**, 426–433
12. Stanley, C. J. & Perham, R. N. (1980) *Biochem. J.* **191**, 147–154
13. Paxton, R. (1988) *Methods Enzymol.* **166**, 313–320
14. Goodwin, G. W., Zhang, B., Paxton, R. & Harris, R. A. (1988) *Methods Enzymol.* **166**, 189–201
15. Harris, R. A., Paxton, R. & Parker, R. A. (1982) *Biochem. Biophys. Res. Commun.* **107**, 1497–1503
16. Srere, P. A. (1969) *Methods Enzymol.* **13**, 3–11
17. Boyer, B. & Odessey, R. (1990) *Biochem. J.* **271**, 523–528
18. Harris, R. A., Paxton, R. & DePaoli-Roach, A. A. (1982) *J. Biol. Chem.* **257**, 13915–13918
19. Solomon, M., Cook, K. G. & Yeaman, S. J. (1987) *Biochim. Biophys. Acta* **931**, 335–338
20. Patston, P. A., Espinol, J. & Randle, P. J. (1984) *Biochem. J.* **222**, 711–719
21. Hutson, N. J., Kerbey, A. L., Randle, P. J. & Sugden, P. H. (1978) *Biochem. J.* **173**, 669–680
22. Wieland, O. H., Siess, E., Schulze-Wethmar, F. H., von Funcke, H. G. & Winton, B. (1971) *Arch. Biochem. Biophys.* **143**, 593–601
23. Holness, M. J., Palmer, T. N. & Sugden, M. C. (1985) *Biochem. J.* **232**, 255–259
24. Harris, R. A., Powell, S. M., Paxton, R., Gillim, S. M. & Nagae, H. (1985) *Arch. Biochem. Biophys.* **243**, 542–555
25. Harris, R. A., Paxton, R., Powell, S. M., Goodwin, G. W., Kuntz, M. J. & Han, A. C. (1986) *Adv. Enzyme Regul.* **25**, 219–237
26. Harris, R. A., Goodwin, G. W., Paxton, R., Dexter, P., Powell, S. M., Zhang, B., Han, A. C., Shimomura, Y. & Gibson, R. (1989) *Ann. N.Y. Acad. Sci.* **273**, 306–316
27. Zhang, B., Paxton, R., Goodwin, G. W., Shimomura, Y. & Harris, R. A. (1987) *Biochem. J.* **246**, 625–631
28. Block, K. P., Aftring, R. P. & Buse, M. G. (1990) *J. Nutr.* **120**, 793–799
29. Caterson, I. D., Fuller, S. J. & Randle, P. J. (1982) *Biochem. J.* **208**, 53–60
30. Vary, T. C., Siegel, J. H., Nakatani, T., Sato, T. & Aoyama, H. (1986) *Am. J. Physiol.* **250**, E634–E640
31. French, T. J., Goode, A. W., Holness, M. J., MacLennan, P. A. & Sugden, M. C. (1988) *Biochem. J.* **256**, 935–939
32. Holness, M. J. & Sugden, M. C. (1989) *Biochem. J.* **258**, 529–533
33. Pettit, F. H., Pelley, J. W. & Reed, L. J. (1975) *Biochem. Biophys. Res. Commun.* **65**, 575–582
34. Kerbey, A. L., Randle, P. J., Cooper, R. H., Whitehouse, S., Pask, H. T. & Denton, R. M. (1976) *Biochem. J.* **154**, 327–348
35. Fatania, H. R., Vary, T. C. & Randle, P. J. (1986) *Biochem. J.* **234**, 233–236
36. Denyer, G. S., Kerbey, A. L. & Randle, P. J. (1986) *Biochem. J.* **239**, 347–354
37. Holness, M. J. & Sugden, M. C. (1987) *Biochem. J.* **247**, 627–634
38. Holness, M. J. & Sugden, M. C. (1990) *Biochem. J.* **268**, 77–81
39. Hutson, S. M. (1988) *J. Nutr.* **118**, 1475–1481
40. Holness, J. J., Liu, Y.-L. & Sugden, M. C. (1989) *Biochem. J.* **264**, 771–776
41. French, T. J., Holness, M. J., McLennan, P. A. & Sugden, M. C. (1988) *Biochem. J.* **250**, 773–779

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