Fuel selection in animals

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I first became interested in biochemistry as a medical student in Cambridge and this led me to take the Part II biochemistry course in 1946–47. I would have been content to be either a biochemist or a physician, but at the critical points in my undergraduate and postgraduate education at Cambridge and at University College Hospital I was encouraged to attempt to be both. The view at the time was that medical research was in need of physicians who were professional scientists. There are comparatively rare individuals who can direct laboratory research at a challenging level from the bedside or who can divide their time between the two activities and succeed at both. I am not one of them. It is necessary for me to do experiments with my own hands in order to have ideas and to advise students and fellows in their work. Because of this I eventually gave up clinical work on appointment as Professor of Biochemistry at Bristol. My present appointment as a Clinical Professor in the Oxford Medical School is perhaps a recognition of the need for scientists in clinical schools — a healthy trend which may be lost if current deprivation continues. The Biochemical Society’s award of the CIBA Medal is an honour which I value and appreciate deeply.

My current research is concerned with the mechanisms which regulate reversible phosphorylation in the mitochondrial pyruvate dehydrogenase (PDH) and branched chain 2-oxoacid dehydrogenase complexes in relation to effects of diabetes and of diet. This lecture is concerned with the PDH complex, its role in fuel selection, and the mechanisms which may mediate effects of diabetes and diet on its activity.

Historical aspects

My interest in carbohydrate metabolism and its regulation was initiated in 1946–47 by Drs. T. R. Mann, D. M. Needham and G. D. Greville, who introduced me to ideas concerning the regulation of glycolysis, the Pasteur effect, energy conservation and the actions of hormones. This interest was fostered and directed towards diabetes by Professor H. P. Himsworth at University College Hospital and later at Cambridge by Professor F. G. Young when I was a Ph.D. student working on the assay of insulin in blood (1952–55). In the period after my Ph.D. I tried various things and then in 1956–57 made two observations that determined the course of much of my subsequent work. The first (a chance observation) was the stimulation by anoxia of glucose uptake by rat diaphragm in vitro. This led on between 1957 and 1962 to the acquisition of evidence that the Pasteur effect in muscle is mediated through activity changes in glucose transport, phosphofructokinase-1 and hexokinase. In 1956–57 also, I read a paper by Drury & Wick (1953), in which they showed that injection of 3-hydroxybutyrate or acetate into rabbits decreases conversion of [14C]glucose into 14CO2. It was possible to show fairly quickly that 3-hydroxybutyrate inhibits glucose utilization in rat diaphragm provided insulin is present. This suggested to me that the ketone body may inhibit intracellular metabolism of glucose and that enhanced oxidation of lipid fuels might have a role in the decreased utilization of glucose by muscle in diabetes or starvation.

Between 1960 and 1967, evidence was obtained with muscle preparations in vitro that effects of starvation and alloxan-diabetes to decrease glucose utilization, glycolysis and pyruvate oxidation in vitro might be mediated by enhanced oxidation of fatty acids formed from endogenous muscle triglyceride. This led to the formulation of the idea of a glucose/fatty acid cycle, the essential components of which were: (1) the relationship between glucose and fatty acid metabolism is reciprocal and not dependent; (2) in vivo, the oxidation of fatty acids and ketone bodies released into the circulation in diabetes or starvation may inhibit the catabolism of glucose in muscle; (3) in vitro, the oxidation of fatty acids released from muscle triglyceride may play a similar role; (4) the effects of fatty acid and ketone body oxidation are mediated by inhibition of the PDH complex, phosphofructokinase-1 and hexokinase; (5) the essential mechanism is an increase in the mitochondrial ratio of [acetyl-CoA]/[CoA], which inhibits the PDH complex and by indirect means leads to inhibition of phosphofructokinase-1 by citrate and of hexokinase by glucose 6-phosphate; (6) the effect of low concentrations of insulin to accelerate glucose transport is inhibited by oxidation of fatty acids or ketone.
bodies. The mechanism of citrate accumulation (unspanning of the citrate cycle) was detailed in 1970. The concept owed much to the parallel work of others, notably H. E. McConnell, W. J. Shipp, and L. H. Opie, and to the discovery of allosteric regulation of phosphofructokinase-I by J. Passamou and O. H. Lowry. [For reviews, including historical aspects of substrate competition for respiration, see Randle et al. (1966, 1970).] Because of technical difficulties these studies did not include measurements of the activity of the PDH complex after extraction from tissues. This was recognized to be an important omission (Randle, 1966) and it was proved to be so when reversible phosphorylation of the PDH complex was discovered by Dr. L. J. Reed and his colleagues (Linn et al., 1969a,b). This discovery and the further discovery of the effect of insulin to activate the PDH complex by dephosphorylation in adipocytes (Coore et al., 1971, Jungas, 1971) made it necessary to examine in more detail the role of lipid fuels as mediators of the effects of starvation and alloxa-diabetes on glucose oxidation.

Fuels: consumption, selection and the role of the PDH complex

In animals the major respiratory fuels are glucose, lipid fuels (fatty acids and ketone bodies) and amino acids, and their storage forms (glycogen, adipocyte and plasma triglyceride, expendable protein). In the fed state the fuel mix depends on diet and in man the average U.K. or American diet gives a mix (in J) of 45–50% carbohydrate, 33–43% fat and 13–17% protein. The proportionate contribution of carbohydrate (which includes glucose derived from amino acids) is overridden by that of fat in starvation or diabetes. Prolonged starvation may reduce the contribution of carbohydrate (in J) to <5% (for reviews, see Cahill & Owen, 1968; Randle et al., 1978). The oxidation of glucose, which accounts for 40% of a 100 g oral glucose load in normal people, is decreased in insulin-dependent diabetics to 13% and in non-insulin-dependent diabetics to 27% (Meyer et al., 1980). The oxidation of glucose in muscle is increased by exercise and may account for approx. 50% of total oxygen consumption during moderate exercise in man. The contribution of glucose oxidation to respiration during exercise is decreased by more prolonged exercise (>1 h), by feeding fat, and by diabetes (see Wahren et al., 1978; Felg & Koivist, 1979; Lefebvre, 1985). Starvation, dietary composition, exercise and diabetes are thus major factors governing the selection of respiratory fuels and/or the rate of respiration. Hormones involved include insulin, glucagon, catecholamines, growth hormone and cortisol.

Glucose recycling (the Cori cycle) is a constant component of carbohydrate metabolism in fed, fasted, exercising or diabetic man or rat. At the enzyme level, recycling depends upon regulation of the PDH complex such that a proportion of lactate, pyruvate and alanine formed from glucose in extrahepatic tissues escapes oxidation, is conveyed to the liver and converted into glucose (Randle et al., 1966). This has been shown rather directly by use of PDH kinase inhibitors such as dichloroacetate which increase the activity of the PDH complex and in consequence decrease circulating lactate, pyruvate and alanine by promoting their oxidation (for review, see Randle et al., 1978). The activity of the PDH complex is the major determinant of glucose oxidation in well-oxygenated tissues in vivo. In some in vitro systems other reactions may be rate-limiting under special circumstances; for example, in hearts perfused for long periods with glucose as the sole substrate, membrane transport may limit oxidation in the absence of added insulin. This does not occur in vivo because lipid fuels are present.

The PDH complex: reactions and chemistry

In animals the PDH complex is mitochondrial and with Mg²⁺, thiamin pyrophosphate (TPP), CoA and NAD catalyses the oxidative decarboxylation of pyruvate into acetyl-CoA, CO₂ and NADH, by a chemically irreversible reaction (Kₑ = 8.4 x 10⁶ at pH 7). The reaction is crucial to glucose conservation in animals which lack other reactions enabling glucose to be re-formed from acetyl-CoA. The PDH holocomplex reaction has a role in ATP synthesis and in the biosynthesis of fatty acids and tri-carboxylate cycle intermediates from glucose. The component enzymes of the complex and their subunit Mr and stoichiometry are shown in Table 1.

PDH complex is resolved by gel filtration at alkaline pH and high ionic strength and yields the E2 core (60 copies of a single subunit), E₁ (αββ) and E₃ (a dimer of a single subunit). PDH kinase is associated with both E₁ and E₂ fractions. There are 60 copies of E₁ (αββ) per mol in pig heart complex but only 30 in ox kidney complex (see Barrera et al., 1972; Sugden & Randle, 1978; Randle, 1981; Reed, 1981; Pettit & Reed, 1982a,b; Kerby & Randle, 1985). A fifth band (band 5 or band X) is detected on SDS/polyacrylamide-gel electrophoresis in Tris buffer (Stanley & Perham, 1980; De Marucci et al., 1985; Kerby & Randle, 1985; Rahmatullah & Roche, 1985). The band migrates close to E₄ and its Mr is either > 55 000 or < 55 000 depending on the assignment of E₃. It has been detected in Western blots after SDS/polyacrylamide-gel electrophoresis of SDS extracts of mitochondria or cells by an antibody to purified ox kidney complex (De Marucci et al., 1985). The function of band 5 is unknown but like E₂ it is acetylated by acetyl-CoA (Rahmatullah & Roche, 1985).

The elementary reactions catalysed by the individual enzymes of the PDH complex are shown in Fig. 1. Non-reversibility of the holocomplex reaction is conferred by decarboxylation (catalysed by E₁); the other reactions are reversible. Lipote attached to α-NH₂ of lysine in the swinging arm of E₂ acts as an acceptor for acetyl and hydrogen and mediates formation of acetyl-CoA (E₂) and NADH, (E₂ and E₃). In the absence of CoA and NAD, acetoin is the principal end-product. In purified Escherichia coli and bovine complexes the E₁ reaction(s) are apparently rate-limiting. Intramolecular coupling of active sites enables a single E₁ component to effect reductive acetylation of a

Table 1. Component enzymes of bovine or pig PDH complex and PDH phosphatase

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Mₛ</th>
<th>Number per mol of complex</th>
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<tbody>
<tr>
<td>E₁: pyruvate dehydrogenase</td>
<td>156 000</td>
<td>30–60</td>
</tr>
<tr>
<td>α-subunit</td>
<td>42 000</td>
<td>60–120</td>
</tr>
<tr>
<td>β-subunit</td>
<td>36 000</td>
<td>60–120</td>
</tr>
<tr>
<td>E₂: dihydrolipopote acetyltransferase (subunit)</td>
<td>52 000</td>
<td>60</td>
</tr>
<tr>
<td>E₃: dihydrolipoyl dehydrogenase</td>
<td>110 000</td>
<td>12</td>
</tr>
<tr>
<td>subunit</td>
<td>55 000</td>
<td>24</td>
</tr>
<tr>
<td>Pyruvate dehydrogenase kinase</td>
<td>93 000</td>
<td>5</td>
</tr>
<tr>
<td>α-subunit</td>
<td>48 000</td>
<td>5</td>
</tr>
<tr>
<td>β-subunit</td>
<td>45 000</td>
<td>5</td>
</tr>
<tr>
<td>Pyruvate dehydrogenase phosphatase</td>
<td>147 000</td>
<td>5</td>
</tr>
<tr>
<td>α-subunit</td>
<td>97 000</td>
<td>50 000</td>
</tr>
</tbody>
</table>
| β-subunit | 50 000 | }

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large number of lipoate residues in the E2 core (Stanley et al., 1981). In purified pig heart complex E2 is apparently rate-limiting as the rate of the holocomplex reaction is increased by further addition of E2 but not of E1 (Kerbey & Randle, 1985). This is presumably due to the greater number of E1 components per mol of pig heart complex (see above).

**End-product inhibition and reversible phosphorylation**

Mammalian PDH complexes are inhibited by acetyl-CoA (competitive with CoA) and NADH, (competitive with NAD) as a result of reduction or reductive acetylation of E1-bound hydroxyethyl-TPP carbanion. End-product inhibition may be important in liver (see later) and as a general means of rapid adjustments of flux. Otherwise, a more important mechanism for mammalian PDH complexes is regulation by reversible phosphorylation (Linn et al., 1969a,b). PDH kinase, an integral component of the complex, phosphorylates E1a with MgATP leading to inactivation of E1 and the holocomplex. Reactions catalysed by E2 and E3 are unaffected (Walsh et al., 1976). Dephosphorylation and reactivation is effected by mitochondrial PDH phosphatase, which is not an integral component of the complex. PDH kinase and PDH phosphatase are each composed of two dissimilar subunits (Table I); kinase activity resides in the a-subunit of PDH kinase (Stepp et al., 1983) and phosphatase activity in the b-subunit of PDH phosphatase (Teague et al., 1982). The function of the other subunits is not known.

In ox and pig complexes phosphorylation is half-site, i.e. equivalent to one a-chain in E1 (a,b). In ox, pig and rat complexes there are three phosphorylation sites which are recovered in two tryptic phosphopeptides (TA, TB) (for sequences see Fig. 2). Sites 1 and 2 may be separated for analysis by cleavage of the Asp-Pro bond in TA with formic acid yielding phosphopeptides TF1 and TF2. Sites 1 and 2 are each inactivating sites. Relative rates of phosphorylation are sites 1 > 2 > 3 for purified complex or in mitochondria. With purified complex or with complex in vivo, phosphorylation of site 1 accounts for >98.5% of inactivation during phosphorylation or in the steady state (Sale & Randle, 1981, 1982a,b). Relative rates of dephosphorylation are sites 2 > 1 > 3 for purified complex or complex in mitochondria (Sale & Randle, 1982a). Phosphorylation of sites 2 and 3 decreases the rate of dephosphorylation of site 1 and of reactivation of the complex by PDH phosphatase (Sugden et al., 1978; Kerbey et al., 1981; Sale & Randle, 1982b). During dephosphorylation of complex in mitochondria occupancy of site 1 accounts for 93% of inactive complex and site 2 for 7% (Sale & Randle, 1982a).

**Fig. 1. Elementary reactions of the PDH complex**

**Fig. 2. Amino acid sequences and seryl phosphorylation sites in tryptic phosphopeptides from fully phosphorylated pig heart PDH complex**

TA, TB, tryptic phosphopeptides; TF1, TF2, cleavage products obtained by incubation at acid pH (formic acid) (Sugden et al., 1979; Sale & Randle, 1981). In ox kidney complex asparagine replaces aspartate at TA8 (Yeaman et al., 1978).
muscle is also decreased by oxidation of fatty acids and/or ketone bodies but the effects are less marked than those of starvation or diabetes (up to 4-fold). In liver oxidation of fatty acids in vitro in short-term experiments is variously reported to decrease per cent active complex or to increase it (e.g. Siess & Wieland, 1976; Dennis et al., 1978). Exercise increases per cent active complex in heart and skeletal muscle; the values attained are lower in starved or diabetic animals. [For general reviews and references to original papers, see Wieland et al. (1973), Randle et al. (1978), Wieland (1983) and McCormack & Denton (1984).]

In vitro, insulin rapidly increases per cent active complex in rat adipose tissue. The hormone has little or no effect in liver and no obvious effect in muscles of fed, starved or alloxan-diabetic rats. Per cent active complex is rapidly increased in heart muscle by inotropic hormones (e.g. β-adrenergic agonists, glucagon) and in liver by glucagon and α-adrenergic agonists (for reviews, see Wieland, 1983; Randle et al., 1984; McCormack, 1985). The effects of starvation or diabetes on per cent active complex are therefore obviously not due to short-term concentration of circulating insulin or of a lower [insulin]/[glucagon] ratio in these conditions. Current evidence indicates that changes in per cent active complex reflect changes in per cent dephosphorylated form of the complex. The mechanisms must, therefore, involve regulation of PDH kinase and/or phosphatase reactions.

**Regulation of PDH kinase and phosphatase reactions by metabolites and cations**

PDH kinase may be assayed by the rate of ATP-dependent inactivation or by the incorporation of 32P from [γ-32P]ATP into the complex or individual sites. PDH phosphatase may be assayed by comparable methods. It is convenient to use reactions which are pseudo-first-order when activity can be expressed as the pseudo-first-order rate constant.

These reactions are ATP-dependent inactivation (provided E1 is rate-limiting for the holocomplex reaction) and dephosphorylation of individual sites. The kinase and phosphatase reactions can be assayed in intact mitochondria where [γ-32P]ATP may be generated in situ from 32P. It is reassuring that the regulatory mechanisms to be described are demonstrable employing intact mitochondria or extracted or purified PDH complex and phosphatase. In mitochondria, PDH complex, kinase and phosphatase utilize only intramitochondrial substrates and effectors.

The PDH phosphatase reaction requires Mg2+ and in the presence of Mg2+, the activity of [γ-32P]ATP may be generated in situ from 32P in the physiological range (0.5–5 mM, Km 0.8 μM), and may be inhibited by NADH, (reversed by NAD) (Linn et al., 1969a,b; Denton et al., 1972; Reed, 1981; Ashour & Hansford, 1983; Fuller & Randle, 1984; McCormack & Denton, 1984; McCormack, 1985).

The PDH kinase reaction is inhibited by ADP (competitive with ATP) and by pyruvate (either non-competitive or uncompetitive with ATP); the inhibition by pyruvate is synergistic with ADP and enhanced by decreasing ratios of [ATP]/[ADP] (Linn et al., 1969a,b; Pratt & Roche, 1979).

The PDH kinase reaction is accelerated by acetyl-CoA and by NADH, and their effects are reversed by CoA and NAD respectively. Activation by acetyl-CoA requires NADH but is not seen when maximal activation has been achieved with NADH. The kinetics have not been fully evaluated. In mitochondria the kinase reaction is therefore regulated by the intramitochondrial ratios of [ATP]/[ADP], [acetyl-CoA]/[CoA] and [NADH]/[NAD] and by intramitochondrial [pyruvate] (Pettit et al., 1975; Hansford, 1976; Kerbey et al., 1976; Roche & Caté, 1976; Kerbey et al., 1977; Cate & Roche, 1978; Ashour & Hansford, 1983; Fuller, 1984; Fuller & Randle, 1984).

There are two hypotheses for the mechanism of the effects of acetyl-CoA, CoA, NADH, and NAD on the rate of the PDH kinase reaction. The allosteric model involves direct interactions with PDH kinase but the evidence is based on work with artificial substrates (Reed, 1981) and has yet to be confirmed (Rahmatullah & Roche, 1985). The alternative model involves covalent modification of the PDH complex initiated by substrate interactions leading to reduction or reductive acetylation of lipoate. This model was first suggested by the observation that the PDH kinase reaction is also activated by low concentrations of pyruvate or by acetoin in the presence but not in the absence of TPP (Cooper et al., 1974). As shown in Fig. 1, pyruvate + TPP, acetoin + TPP, and acetyl-CoA + NADH all lead to reductive acetylation of lipoate. This model was supported further by the dependence of the acetyl-CoA effect upon NADH, and by evidence that other reducing agents (dithiothreitol or dihydroxyacetone) may replace NADH (Cooper et al., 1975; Roche & Caté, 1976; Caté & Roche, 1978, 1979). It has been further supported by evidence that activation of PDH kinase by acetyl-CoA is correlated with acetylation of the complex as opposed to the concentration of acetyl-CoA (Rahmatullah & Roche, 1985).

In what follows I shall concentrate on the molecular mechanisms which lead to phosphorylation and inactivation of the complex in rats starved for 48 h or in which diabetes has been induced by alloxan. The mechanism of action of insulin in adipocytes is not known but it is apparently due to activation of PDH phosphatase (Huges & Denton, 1976) possibly by uncharacterized mediators (for reviews see Jarett et al., 1982; Larner et al., 1982). There is convincing evidence that the effects of exercise in muscles and the short-term effects of glucagon and of α-adrenergic agonists in liver are mediated mainly by activation of PDH phosphatase by Ca2+ (McCormack & Denton, 1984; McCormack, 1985).

**Molecular mechanisms mediating effects of starvation and diabetes on PDH interconversion in muscles**

In muscles the effect of fatty acids and/or ketone bodies to decrease per cent active PDH complex is a result of activation of PDH kinase by the increase in mitochondrial [acetyl-CoA]/[CoA] ratio occasioned by their oxidation. Thus the effects of fatty acids on both ratios are reversed by β-oxidation inhibitors (2-tetradecylglycinate, phenylxoritate carboxylate) and the increase in [acetyl-CoA]/[CoA] ratio effected by oxidation of fatty acids or ketone bodies is over the range required for maximum activation of PDH kinase (Randle et al., 1970; Kerbey et al., 1979; Caterson et al., 1982; Fuller, 1984). In heart muscle, β-oxidation of fatty acids is necessary for the effect of starvation and diabetes on per cent active PDH complex because tetradecylglycinate can reverse their effects by restoring normal ratios of [acetyl-CoA]/[CoA] (Caterson et al., 1982).

In heart and skeletal muscle, enhanced oxidation of fatty acids and ketone bodies is not the only mechanism involved in the effects of starvation and diabetes on per cent PDH complex. This was first suggested by the long latency (24–48 h) of effects of starvation and diabetes and by the observation that their effects on per cent active complex are greater than those of fatty acids and ketone bodies. The operation of other mechanisms was shown directly when it was discovered that the effect of starvation or diabetes to lower per cent active complex persists in muscle mitochondria incubated in vitro with 2-oxoglutarate + l-malate (Kerbey et al., 1976; Fuller & Randle, 1984). Direct analysis showed that the effects of diabetes and starvation persisting in mitochondria were not associated with increased ratios.
Table 2. Effect of starvation or alloxan-diabetes on PDH kinase activity in mitochondrial extracts: apparent first-order rate constants for MgATP-dependent inactivation

Results are means \( \pm \) S.E.M. \( P < 0.001 \) for effects of starvation or diabetes.

<table>
<thead>
<tr>
<th>Tissue of origin of mitochondria</th>
<th>PDH kinase activity (min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fed</td>
</tr>
<tr>
<td>Heart</td>
<td>0.43 ( \pm ) 0.04</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>0.51 ( \pm ) 0.02</td>
</tr>
<tr>
<td>Liver</td>
<td>0.89 ( \pm ) 0.04</td>
</tr>
<tr>
<td>Cultured hepatocytes*</td>
<td>0.81 ( \pm ) 0.03</td>
</tr>
</tbody>
</table>

* Cultured for 21 h in medium 199 (5.5 mM-glucose + 0.6 mM-nicotinamide acetate).

Table 3. PDH kinase activity in rat liver mitochondrial extracts: apparent first-order rate constants for ATP-dependent inactivation

<table>
<thead>
<tr>
<th>Fraction(s)</th>
<th>PDH kinase activity (min(^{-1}))</th>
<th>Fraction</th>
<th>Fed</th>
<th>Starved</th>
<th>Ratio (starved/fed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfractionated</td>
<td>1.4 ( \pm ) 0.06</td>
<td>2.9 ( \pm ) 0.1</td>
<td>2.2 ( \pm ) 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction 1 (PDH complex)</td>
<td>0.2 ( \pm ) 0.02</td>
<td>1.2 ( \pm ) 0.06</td>
<td>5.0 ( \pm ) 0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fractions (1 + 2)</td>
<td>2.3 ( \pm ) 0.12</td>
<td>5.6 ( \pm ) 0.24</td>
<td>2.4 ( \pm ) 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precipitate, pH 6.5, 5% poly(ethylene glycol)</td>
<td>2.9 ( \pm ) 0.10</td>
<td>6.4 ( \pm ) 0.30</td>
<td>2.2 ( \pm ) 0.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
complex in liver although it was effective in heart and kidney (Caterson et al., 1982). The potential effect to increase per cent active complex may have been nullified by impaired acetacetate/pyruvate exchange.

The unpublished results of experiments undertaken with G. S. Denyer and A. L. Kerbey pertaining to stable activation of rat liver PDH kinase by starvation are shown in Tables 3 and 4. Results for liver mitochondrial extracts were comparable with those for heart and skeletal muscle (Table 2) and the effects of gel filtration (Table 3) were also comparable with those for heart. The activity of PDH kinase after recombination of fractions 1 and 2 was greater than in extracts (heart or liver) and this is attributed to removal by gel filtration of low-M<sub>c</sub> inhibitors of PDH kinase such as TPP and ADP. Activities comparable with those observed after recombination of fractions 1 and 2 were seen when complex was precipitated from extracts with poly(ethylene glycol), suggesting co-precipitation, i.e. tight binding of kinase activator protein to the complex. The results of cross-over experiments (Table 4) were particularly illuminating because they suggest that the fed/starved difference in PDH kinase activity is conferred by the activity of fraction 2 and not by the activity of PDH kinase intrinsic to PDH complex (fraction 1).

There are two observations which at first sight might appear to be at variance with this conclusion, but they may be accommodated if it is assumed that starvation increases the affinity of fraction 2 for PDH complex. These are the high starved/fed ratio for fraction 1 after gel filtration (Table 4, line 1) and the persistence of a ratio > 1 when fraction 1 from fed and starved is mixed with fraction 2 from fed (Table 4, line 2). Evidence in support of this assumption has been obtained in experiments in which purified pig heart complex was mixed with kinase activator protein fractions from fed and starved rats and subjected to gel filtration. The measured PDH kinase activities of the pig heart PDH complex before gel filtration and in fraction 1 after gel filtration were consistent with this interpretation (results not shown).

The results of preliminary experiments have shown also that the effect of starvation on fraction 2 is to increase the V<sub>max</sub> of the PDH kinase reaction.

These results suggest the following hypothesis. The activity of PDH kinase is increased by a protein (or proteins) which is removed, in whole or in part, when PDH complex is gel-filtrated (or presumably when sedimented). The specific activity of this protein fraction is increased by starvation through a stable mechanism such as covalent modification. If the hypothesis is correct then it has a number of interesting implications. It could explain the variability in kinase activity between different preparations of purified PDH complex (variable activities of protein activator of PDH kinase in slaughterhouse material). It could explain the loss of kinase activity at very low concentrations of PDH complex or after sedimentation of PDH complex at low concentration which is reversed by addition of the activator protein (dissociation of bound activator protein) (Kerbey & Randle, 1982). It is currently held that phosphorylation of E1 is facilitated by binding of PDH kinase to E2; consideration should now be given to the possibility that the effect of E2 may be due to associated activator protein.

Long-term culture of hepatocytes

We have embarked upon long-term culture of hepatocytes prepared from rat liver by collagenase digestion because it may provide information about the hormonal and nutritional factors responsible for the stable increase in PDH kinase activity induced by starvation and diabetes, the factors involved in their reversal by refeeding or insulin treatment, and about mechanisms. The results are promising and have shown that 21 h of culture with glucagon (55 nM) and sodium n-octanoate (1 mM) can effect a 250% increase in PDH kinase activity and a 34% decrease in per cent active complex following after the expected initial increase (Fatania et al., 1986). The main results are summarized in Fig. 3.

Multi-site phosphorylation in vivo

As mentioned previously, phosphorylation of sites 2 and 3 in the E1a component of PDH complex decreases the rate of reactivation and the rate of dephosphorylation of site 1 by PDH phosphatase. It was of interest therefore to determine the effects of starvation and diabetes on site occupancies; and the relationship between per cent inactive complex and the occupancy of the three sites in vivo. This has been accomplished with heart mitochondria and in the heart in vivo (Sale & Randle, 1982a,b). Occupancy of site 1 was correlated linearly with per cent inactive complex; occupancy-
Inhibitors of (A) lipolysis, (B) fatty acid oxidation and (C) PDH kinase.

Fig. 4. Model for the mechanism of the effect of starvation and alloxan-diabetes to reduce per cent active PDH complex in rat tissues.

Inhibitors of (A) lipolysis, (B) fatty acid oxidation and (C) PDH kinase.

cies of sites 2 and 3 in inactive complex were 56% and 39% respectively when per cent inactive complex was less than approximately 70% (i.e. at or below the level for resting fed normal rats). The occupancy of sites 2 and 3 increased as per cent inactive complex increased above 70% to approximate to full occupancy and equivalence to site 1 at 98-99% inactive complex (the level for starved or diabetic rats). The relative rates of reactivation by phosphatase of phosphorylated complex from starved and fed rats were 2.8/1 (fed/starved).

General conclusions

Our current views concerning the mechanism by which starvation or diabetes increase the phosphorylation and inactivation of the PDH complex together with the sites of action of drugs which may reverse the process are summarized in the model depicted in Fig. 4. This is a kinase-directed model, PDH kinase being activated by products of oxidation of lipid fuels and by a stable mechanism involving an activator protein or proteins. For the purpose of hypothesis the effects of starvation and diabetes on the protein activator are assumed to involve covalent modification. Long-term culture has suggested a role for lipid fuels and hormones in the modification. The stable mechanism of activation of PDH kinase, and consequential effects on multi-site phosphorylation, induced by starvation or diabetes change the reference point from which other effectors exert their effect. As a consequence per cent active complex remains at a lower level when, for example, PDH kinase is activated by Ca\(^{2+}\) during muscle contraction, or when PDH kinase is inhibited by ADP. The stable mechanism of PDH kinase activation might also function as a hysteresis mechanism during starvation, serving to delay full restoration of PDH complex activity when feeding is resumed. It has not escaped our attention that the protein factor which activates PDH kinase might have opposing effects on the PDH phosphatase reaction. There is evidence that reactivation of inactivated PDH complex by phosphatase in heart mitochondria is inhibited by starvation of diabetes; this is presently attributed to multi-site phosphorylation (Hutson et al., 1978).

Further progress awaits purification and characterization which may now be practicable with the liver system.

It is difficult to express adequately in words the debt owed to students, postdoctoral and visiting fellows, and technicians for their contribution to this project, because the debt is so large. Nor in a project spanning some 20 years is it possible to name them all. In more recent work I am particularly indebted to Drs. A. L. Kerby, H. R. Fatania, S. J. Fuller, G. J. Sale and T. C. Vary, Mr. G. S. Denyer, Ms. Caroline Philips and Ms. Lynne Richardson. This work has been made possible by support from the British Diabetic Association and the Medical Research Council.


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