Regulation of Pyruvate Dehydrogenase in Rat Heart

MECHANISM OF REGULATION OF PROPORTIONS OF DEPHOSPHORYLATED AND
PHOSPHORYLATED ENZYME BY OXIDATION OF FATTY ACIDS AND KETONE BODIES AND OF
EFFECTS OF DIABETES: ROLE OF COENZYME A, ACETYL-COENZYME A AND
REDUCED AND OXIDIZED NICOTINAMIDE-ADENINE DINUCLEOTIDE

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1. The proportion of active (dephosphorylated) pyruvate dehydrogenase in perfused rat heart was decreased by alloxan-diabetes or by perfusion with media containing acetate, *n*-octanoate or palmitate. The total activity of the dehydrogenase was unchanged. 2. Pyruvate (5 or 25 mM) or dichloroacetate (1 mM) increased the proportion of active (dephosphorylated) pyruvate dehydrogenase in perfused rat heart, presumably by inhibiting the pyruvate dehydrogenase kinase reaction. Alloxan-diabetes markedly decreased the proportion of active dehydrogenase in hearts perfused with pyruvate or dichloroacetate. 3. The total activity of pyruvate dehydrogenase in mitochondria prepared from rat heart was unchanged by diabetes. Incubation of mitochondria with 2-oxo-glutarate plus malate increased ATP and NADH concentrations and decreased the proportion of active pyruvate dehydrogenase. The decrease in active dehydrogenase was somewhat greater in mitochondria prepared from hearts of diabetic rats than in those from hearts of non-diabetic rats. Pyruvate (0.1–10 mM) or dichloroacetate (4–50 μM) increased the proportion of active dehydrogenase in isolated mitochondria presumably by inhibition of the pyruvate dehydrogenase kinase reaction. They were much less effective in mitochondria from the hearts of diabetic rats than in those of non-diabetic rats. 4. The matrix water space was increased in preparations of mitochondria from hearts of diabetic rats. Dichloroacetate was concentrated in the matrix water of mitochondria of non-diabetic rats (approx. 16-fold at 10 μM); mitochondria from hearts of diabetic rats concentrated dichloroacetate less effectively. 5. The pyruvate dehydrogenase phosphate phosphatase activity of rat hearts and of rat heart mitochondria (approx. 1–2 munit/unit of pyruvate dehydrogenase) was not affected by diabetes. 6. The rate of oxidation of [1-¹⁴C]pyruvate by rat heart mitochondria (6.85 nmol/min per mg of protein with 50 μM-pyruvate) was approx. 46% of the $V_{max.}$ value of extracted pyruvate dehydrogenase (active form). Palmitoyl-L-carnitine, which increased the ratio of [acetyl-CoA]/[CoA] 16-fold, inhibited oxidation of pyruvate by about 90% without changing the proportion of active pyruvate dehydrogenase. It is concluded that inhibition of pyruvate dehydrogenase by the increased ratio of [acetyl-CoA]/[CoA] did not enhance phosphorylation and inactivation of the dehydrogenase under these conditions, perhaps because the kinase was fully activated by the high ratio of [NADH]/[NAD⁺] (see point 8). 7. A wide variety of respiratory substrates increased ATP concentration and lowered pyruvate dehydrogenase activity (active form) in rat heart mitochondria. In general ATP concentration and pyruvate dehydrogenase activity were inversely correlated, with the following important exceptions. Succinate (0.25 or 5 mM) generated equivalent ATP concentrations, but pyruvate dehydrogenase activity was lowest at 5 mM. As palmitoylcarnitine concentration was increased from 10 to 50 μM, pyruvate dehydrogenase activity fell, whereas ATP concentration was little changed. Octanoate (50 μM) decreased pyruvate dehydrogenase
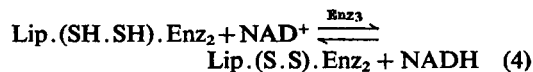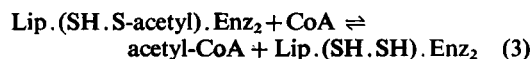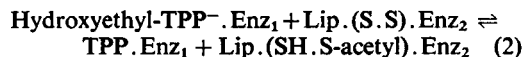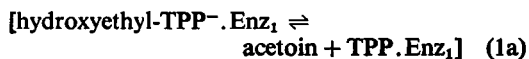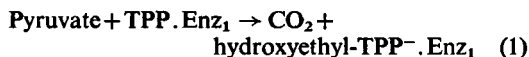
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activity substantially, whereas ATP concentration increased only moderately; the effects of octanoate on ATP concentration and dehydrogenase activity were blocked by oligomycin. These observations suggested that factors other than ATP concentration may regulate the phosphorylation and inactivation of pyruvate dehydrogenase in rat heart mitochondria. 8. The pig heart pyruvate dehydrogenase kinase reaction, assayed by incorporation of ^{32}P into protein-bound phosphate from $[\gamma\text{-}^{32}P]ATP$, was activated by NADH $(5\,\mu M\text{-}2\,mM)$. The effect of NADH was reversed by NAD^+, which had no effects alone. In the presence of non-stimulating or partially stimulating mixtures of NAD^+ plus NADH, acetyl-CoA $(0.25\,mM)$ increased the rate of the kinase reaction. CoA $(0.25$ or $2\,mM)$ inhibited the kinase reaction and with mixtures of NAD^+ plus NADH; acetyl-CoA reversed the inhibition by CoA. It is concluded that the activity of pyruvate dehydrogenase kinase is enhanced by increasing ratios of [NADH]/[NAD^+] and of [acetyl-CoA]/[CoA]. No consistent significant effects of NAD^+, NADH, CoA or acetyl-CoA on the pyruvate dehydrogenase phosphate phosphatase reaction were detected. 9. The concentrations of NADH, NAD^+, acetyl-CoA and CoA have been measured in rat heart mitochondria incubated without substrate or with oxoglutarate plus malate, or with n-octanoate, or with palmitoyl-L-carnitine plus malate, or with succinate. The results of these measurements in conjunction with measurements of ATP concentration and of pyruvate dehydrogenase activity (active form) provide evidence for effects of the ratios of [NADH]/[NAD^+] and of [acetyl-CoA]/[CoA] on the pyruvate dehydrogenase kinase reaction in rat heart mitochondria. 10. Since ratios of [NADH]/[NAD^+] and of [ATP]/[ADP] may be related through equilibrium considerations in respiratory-chain phosphorylation, earlier conclusions about the role of [ATP]/[ADP] ratios in the regulation of phosphorylation and dephosphorylation of pyruvate dehydrogenase ware incomplete. Coupling of the two ratios through the respiratory chain and respiratory-chain phosphorylation may lead to an amplification effect in the regulation of pyruvate dehydrogenase. 11. It was concluded in a previous study that inhibitory effects of fatty acids and ketone bodies on the oxidation of pyruvate in rat heart may be mediated by the increased ratio of [acetyl-CoA]/[CoA], which inhibits pyruvate dehydrogenase [Garland & Randle (1964a) *Biochem. J.* **91**, 6c–7c]. The present study has added further evidence in support of this view by extending the role of the ratio of [acetyl-CoA]/[CoA] to the phosphorylation and inactivation of pyruvate dehydrogenase. The role of mitochondrial [NADH]/[NAD^+] ratio in the regulation of pyruvate dehydrogenase in rat heart has yet to be clearly defined.

Pyruvate oxidation in perfused rat heart is inhibited by alloxan-diabetes and by the oxidation of fatty acids or of ketone bodies (Evans *et al.*, 1963; Williamson, 1964; Garland *et al.*, 1964). The release and oxidation of fatty acids is enhanced in perfused heart and may be responsible for the inhibition of pyruvate oxidation in diabetes (Garland & Randle, 1964b; Denton & Randle, 1967a,b). These findings have generally been interpreted in terms of inhibition of pyruvate dehydrogenase, although the effects of fatty acids and of ketone bodies on pyruvate permeability have not been excluded.

Pyruvate dehydrogenase (EC 1.2.4.1) catalyses the conversion of pyruvate, CoA and NAD^+ into acetyl-CoA, NADH and CO_2 in the presence of thiamin pyrophosphate (TPP) and Mg^{2+} by reactions (1)–(4) below (Gunsalus, 1954; Reed, 1960; Ullrich & Mannschreck, 1967; Schreiber *et al.*, 1963).

$$Pyruvate + TPP.Enz_1 \rightarrow CO_2 + \\ hydroxyethyl\text{-}TPP^-.Enz_1 \quad (1)$$

$$[hydroxyethyl\text{-}TPP^-.Enz_1 \rightleftharpoons \\ acetoin + TPP.Enz_1] \quad (1a)$$

$$Hydroxyethyl\text{-}TPP^-.Enz_1 + Lip.(S.S).Enz_2 \rightleftharpoons \\ TPP.Enz_1 + Lip.(SH.S\text{-}acetyl).Enz_2 \quad (2)$$

$$Lip.(SH.S\text{-}acetyl).Enz_2 + CoA \rightleftharpoons \\ acetyl\text{-}CoA + Lip.(SH.SH).Enz_2 \quad (3)$$

$$Lip.(SH.SH).Enz_2 + NAD^+ \overset{Enz_3}{\rightleftharpoons} \\ Lip.(S.S).Enz_2 + NADH \quad (4)$$

where Lip.(S.S), Lip.(SH.S-acetyl) and Lip.(SH.SH) are lipoate, acetylhydrolipoate and dihydrolipoate respectively.

The three enzymes catalysing the complete reaction sequence (Enz$_1$, pyruvate decarboxylase; Enz$_2$, dihydrolipoate acetyltransferase; Enz$_3$, dihydrolipoyl dehydrogenase) are bound together in a single complex. Interactions of the three enzymes involved in reactions (2)–(4) is effected by lipoate bound covalently to the acetyltransferase (Enz$_2$), which visits the active site of the three enzymes sequentially (Reed, 1969). If reactions (3) and (4) are blocked (i.e. in the absence of CoA and NAD^+), pyruvate is decarboxylated and side products, principally acetoin, are formed by reactions (1) and

(1a); lipoate is also acetylated by reaction (2). Acetoin may also be formed from acetyl-CoA and NADH by reversal of reactions (4) to (1a), which are reversible (Schreiber *et al.*, 1963; Walsh *et al.*, 1976).

Mammalian pyruvate dehydrogenases are inhibited by acetyl-CoA (competitive with CoA), by NADH (competitive with NAD^+) and by acetoin (competitive with pyruvate) (Garland & Randle, 1964a; Wieland *et al.*, 1969; Bremer, 1969; Tsai *et al.*, 1973; Cooper *et al.*, 1974). The mechanism of inhibition by acetyl-CoA plus NADH may involve acetylation of lipoate by reactions (4) to (2) (Garland, 1964). The substantial rise in the ratio [acetyl-CoA]/[CoA] induced by fatty acids, ketone bodies and by alloxan-diabetes may mediate their inhibitory effects on pyruvate dehydrogenase in rat heart (Garland & Randle, 1964a,b). With acetate, for example, the ratio [acetyl-CoA]/[CoA] can increase some 60-fold and the oxidation of pyruvate is almost completely suppressed within 1 min of entry of the substrate into the coronary circulation (Randle *et al.*, 1970). The role of the mitochondrial $[NADH]/[NAD^+]$ ratio in the regulation of pyruvate dehydrogenase by fatty acid or by ketone-body oxidation is not known.

Linn *et al.* (1969a,b) showed that the mammalian pyruvate dehydrogenase complex is regulated by phosphorylation and dephosphorylation. Pyruvate dehydrogenase kinase (tightly bound to the complex) catalyses the phosphorylation and inactivation of pyruvate decarboxylase with $ATPMg^{2-}$; a phosphatase (less tightly bound) catalyses the dephosphorylation and reactivation. In rat heart, alloxan-diabetes or perfusion with fatty acids or with ketone bodies stimulates the phosphorylation and inactivation of pyruvate dehydrogenase (Wieland *et al.*, 1971a,b; Whitehouse *et al.*, 1974). The mechanism of this effect is not known, but it presumably involves activation of the kinase reaction or the inhibition of the phosphatase reaction or both.

The kinase reaction was known to be inhibited by ADP and by pyruvate and the phosphatase reaction was known to require Mg^{2+} and Ca^{2+} (Linn *et al.*, 1969a,b; Denton *et al.*, 1972). Further studies showed that pyrophosphate compounds, Mg^{2+} and Ca^{2+} were also inhibitors of the kinase reaction (Cooper *et al.*, 1974). However, these observations did not provide an obvious explanation for effects of diabetes, fatty acids and ketone bodies on the phosphorylation and inactivation of pyruvate dehydrogenase. It has always seemed likely that the [acetyl-CoA]/[CoA] and $[NADH]/[NAD^+]$ ratios might mediate the effects of fatty acids and of ketone bodies on the phosphorylation of pyruvate dehydrogenase in addition to their inhibitory effect on the dehydrogenase. Initial attempts at demonstrating effects of acetyl-CoA on the kinase reaction gave rather inconsistent effects (Cooper *et al.*, 1974). The studies of Cooper *et al.* (1974) did, however, show that low

concentrations of pyruvate or of acetoin activated the kinase reaction in the presence of thiamin pyrophosphate but not in its absence. However, evidence for the activation of the kinase reaction in isolated mitochondria by pyruvate or by acetoin could not be obtained. In studies by Walsh *et al.* (1976) it was shown that acetoin is slowly metabolized by pyruvate dehydrogenase (K_m 0.5 mM) through reactions (1a) (in reverse) plus (2)–(4). These observations suggested that activation of the kinase by pyruvate at low concentration or by acetoin could involve enzyme-bound acetylhydrolipoate or enzyme-bound hydroxyethyl-TPP^- through reactions (1), (1a) (in reverse) and (2). If this was so then acetyl-CoA plus NADH should also activate the kinase by reversal of reactions (4)–(2), and such effects were not consistently seen. The studies of Walsh *et al.* (1976) also showed that 2-mercaptoethanol added to pyruvate dehydrogenase preparations could reduce enzyme-bound lipoate. It seemed possible that NADH might itself activate the kinase reaction in preparations only if the lipoate was oxidized and that in order to demonstrate the effects of acetyl-CoA suitable mixtures of NADH and NAD^+ would be necessary. This expectation has been realized in the present study, where it is shown that NADH activates the kinase reaction in a dehydrogenase preparation in which lipoate was largely oxidized, that NAD^+ reverses NADH activation, and that with mixtures of NAD^+ plus NADH which produce only slight activation, acetyl-CoA activates the kinase reaction and CoA inhibits it. The study also includes a detailed appraisal of effects of diabetes and of fatty acid metabolism on the phosphorylation and inactivation of pyruvate dehydrogenase in rat heart and rat heart mitochondria.

Experimental

Materials

Biochemical reagents and enzymes were purchased from Boehringer Corp. (London) Ltd., London W.5, U.K., or from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K., with the following exceptions. Crystalline insulin was a gift from Boots Pure Drug Co. Ltd., Nottingham, U.K. Bovine plasma albumin (fraction V, Sigma) was defatted by the method of Chen (1967). Alloxan (Kodak Ltd., Kirkby, Liverpool, U.K.) was recrystallized from hot water. Palmitic acid was purchased from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.; it was bound to plasma albumin and solutions were prepared for perfusion as described by Garland *et al.* (1964). L-Carnitine chloride was obtained from Koch–Light Laboratories, Colnbrook, Bucks., U.K. Acetyl-L-carnitine was synthesized by the method of Fraenkel & Friedman (1957) and palmitoyl-L-carnitine was synthesized by the method of Bremer (1962); they

were substantially free of L-carnitine [<1%, w/w; assay as given by McAllister et al. (1973)] and contained more than 98% of the expected amount of esterified carnitine (assayed as L-carnitine after alkaline hydrolysis).

Radiochemicals (^3H_2O, [1-^{14}C]pyruvate, [^{32}P]P_i and [U-^{14}C]sucrose) were from The Radiochemical Centre, Amersham, Bucks., U.K. [γ-^{32}P]ATP was made by the method of Glynn & Chappell (1964) or was purchased from The Radiochemical Centre. Dichloro[2-^3H]acetic acid was prepared from ^3H_2O and trichloroacetic acid by copper reduction (Doughty & Derge, 1931) by Mr. Peter Crossley. The crude material was freed of Cu^{2+} by passage through Dowex-50 (H^+ form), Cl^- was removed with Ag_2CO_3 and Ag^{2+} was removed by passage through Dowex-50 (H^+ form). The dichloroacetic acid was then converted into the di-isopropylammonium salt by the addition of excess of di-isopropylamine and ^3H_2O was removed by freeze-drying, which was repeated (after solution in water) until the distillate was free of ^3H_2O. Dichloroacetic acid was then regenerated by passage through Dowex-50 (H^+ form) and its purity checked by chromatography on silica-gel plates in methyl ethyl ketone containing 0.25% (v/v) formic acid, with marker spots of the three chloroacetic acids. The final product had a specific radioactivity of 10.6 mCi/mmol and contained less than 1% of monochloroacetic acid, trichloroacetic acid or ^3H_2O (1% was the limit of detection).

Pyruvate dehydrogenase was purified from pig heart by a modification of the method of Linn et al. (1972) as given by Cooper et al. (1974). Pyruvate dehydrogenase [^{32}P]phosphate and pig heart pyruvate dehydrogenase phosphate phosphatase were prepared as described by Denton et al. (1972). 2-Oxoglutarate dehydrogenase was obtained as a by-product from the preparation of pyruvate dehydrogenase. The precipitate obtained at pH 6.5 with 1.5–2% polyethylene glycol contains the 2-oxoglutarate dehydrogenase; the pellets were redissolved in 20 mM-potassium phosphate buffer, pH 7, containing 1 mM-dithiothreitol, and the 2-oxoglutarate dehydrogenase was removed by centrifuging for 2 h at 150000$g_{av.}$, redissolved in the same buffer (at 20 units/ml) and stored at $-10°C$. Arylamine acetyltransferase (EC 2.3.1.5) was purified from pigeon liver by the method of Tabor et al. (1953).

Methods

Heart perfusions. Hearts were obtained from male albino Wistar rats (300–400 g) which had free access to water and to diet 41B (Oxoid, London S.E.1, U.K.). Rats were injected intraperitoneally with heparin (100 units, Sigma) and Nembutal (60 mg/kg) to prevent blood clotting and to induce anaesthesia before removal of the heart. Alloxan-diabetes was induced by the injection of alloxan (60 mg/kg body

wt.) into a tail vein under diethyl ether anaesthesia and was confirmed 40–48 h later by testing the concentration of blood glucose with Dextrostix (Ames Co., Miles Laboratories, Slough, Bucks., U.K.) at the time of removal of the heart. Hearts were perfused at 38°C by drip-through (or by recirculation in experiments with media containing bovine plasma albumin) with bicarbonate-buffered saline medium (Krebs & Henseleit, 1932) gassed with O_2+CO_2 (95:5) with the additions and for the time given in the text, Tables or Figures. The perfusion pressure was 8.0 kPa (60 mmHg). At the end of the perfusion period the heart was frozen with a tissue clamp pre-cooled in liquid N_2. Extracts were prepared for the assay of pyruvate dehydrogenase and of pyruvate dehydrogenase phosphate phosphatase as described by Whitehouse & Randle (1973) with the further addition of 5 mM-2-mercaptoethanol to the extraction medium.

Heart mitochondria. For preparation of mitochondria rat hearts were removed under Nembutal anaesthesia (see above). This was essential for the preparation of well-coupled mitochondria from hearts of alloxan-diabetic rats and was used as a routine. The remaining operations were carried out at 2°C. The heart was cut open and washed free of blood with sucrose medium (0.25 M-sucrose/5 mM-Tris/HCl/2 mM-EGTA*, pH 7.4). Each heart was then cut up with scissors in 5 ml of sucrose medium and disrupted either with a manual Teflon–glass Potter–Elvehjem homogenizer or by means of a 3 s burst with a Polytron PT 10 homogenizer (see Severson et al., 1976). The mitochondria were separated and washed with (in succession) a low-speed centrifugation (500$g_{av.}$ for 10 min) and three high-speed centrifugations (9000$g_{av.}$ for 10 min) and taken up in sucrose medium and diluted to 20 mg of protein/ ml [protein was determined by the method of Gornall et al. (1949)]. The use of the Polytron homogenizer was very convenient and doubled the yield of mitochondria to approx. 10 mg of mitochondrial protein per heart. The respiratory-control ratio (Chappell & Hansford, 1965) with either method of preparations was between 5 and 12 for mitochondria from hearts of non-diabetic rats and between 3.5 and 4.5 for diabetic rats (measured with 0.5 mM-L-malate, 5 mM-2-oxoglutarate and 0.25 mM-ADP).

Mitochondria were incubated in KCl medium (0.12 M-KCl/20 mM-Tris/HCl/5 mM-potassium phosphate buffer, pH 7.4/2 mM-EGTA) at 30°C. O_2 consumption was measured with a Clark oxygen electrode by using 4 ml of KCl medium and approx. 1–1.5 mg of mitochondrial protein.

For the assay of activities of pyruvate dehydro-

* Abbreviation: EGTA, ethanedioxybis(ethylamine)-tetra-acetate.

genase, pyruvate dehydrogenase phosphate phosphatase and citrate synthase, mitochondria were incubated in KCl medium in Eppendorff centrifuge tubes. Details of the time of incubation and the additions are given in the text, Tables or Figures. At the end of the incubation, mitochondria were separated by centrifuging for 20s in an Eppendorff 3200 centrifuge. The supernatant was aspirated and the mitochondrial pellet frozen in liquid N_2 (less than 10s). For the assay of active pyruvate dehydrogenase and of citrate synthase the frozen pellet was dispersed at $-10°C$ with an ultrasonic disintegration probe into cold ($-15°C$) aq. 50% (v/v) glycerol containing 50mM-Tris/HCl, 5mM-EDTA, 1mM-dithiothreitol, pH7.0 (0.5ml/mg of mitochondrial protein). For the assay of total pyruvate dehydrogenase or of pyruvate dehydrogenase phosphate phosphatase mitochondria were extracted by ultrasonic disintegration in the phosphate medium of Whitehouse et al. (1974).

For determination of mitochondrial spaces of ^3H_2O, [U-^{14}C]sucrose and dichloro[2-^{14}C]acetate, mitochondria were incubated in KCl medium in Eppendorff centrifuge tubes for the times and with the additions given in the text, Tables or Figures. At the end of incubation, mitochondria were separated by centrifuging for 20s in an Eppendorff 3200 centrifuge and the supernatant was aspirated and retained for assay of radioactivity. The mitochondrial pellet was frozen in liquid N_2, thawed, dispersed by aspiration with a syringe into 0.1ml of KCl medium and transferred with one wash (0.1ml) into methoxyethanol/toluene scintillator (Severson et al., 1974) for assay of radioactivity.

For the assay of ATP, NAD$^+$, CoA and acetyl-CoA, mitochondria were incubated in Eppendorff centrifuge tubes and the incubation was terminated by the addition of $20\mu l$ of 70% (v/v) HClO$_4$/ml of incubation medium. After cooling at 0°C, KClO$_4$ and precipitated protein was separated by centrifugation.

For the assay of NADH the mitochondrial incubation was terminated by the addition of 0.6vol. of 1M-KOH in ethanol (La Noue et al., 1973).

Enzyme assays. Pyruvate dehydrogenase in extracts of heart or heart mitochondria (active, dephosphorylated form) was assayed spectrophotometrically by coupling to arylamine acetyltransferase (EC 2.3.1.5) as described by Coore et al. (1971). Total pyruvate dehydrogenase (the sum of the active, dephosphorylated form plus the inactive, phosphorylated form) was assayed similarly in extract of heart or heart mitochondria after incubation with pyruvate dehydrogenase phosphate phosphatase (Whitehouse et al., 1974). Pyruvate dehydrogenase phosphate phosphatase was assayed in extracts of heart or heart mitochondria with pyruvate dehydrogenase [^{32}P]phosphate by the phosphatase assay described by Randle et al. (1974). Pyruvate dehydrogenase kinase was assayed by the radioactivity method

by using [γ-^{32}P]ATP as described by Cooper et al. (1974). The preparations of pig heart pyruvate dehydrogenase used in this study showed a close correlation between [^{32}P] phosphate incorporation and loss of pyruvate dehydrogenase activity. Citrate synthase was assayed in extracts of heart or of heart mitochondria by a modification of the method of Srere et al. (1963) as given by Coore et al. (1971).

Production of $^{14}CO_2$ from [1-^{14}C]pyruvate. Mitochondria were incubated in KCl medium (containing [1-^{14}C]pyruvate) in 20ml glass scintillation bottles, sealed with skirted rubber caps and containing a small glass tube with 0.1ml of 1M-Hyamine in methanol. The incubation was initiated by the addition of mitochondria. The incubation was terminated by the addition of 0.2ml of 70% (v/v) HClO$_4$ and the $^{14}CO_2$ liberated was collected in Hyamine by a further 60min of incubation. Radioactivity was assayed in toluene-based scintillator (Severson et al., 1974).

Assay of metabolites. ATP in $10\mu l$ of HClO$_4$ extracts of mitochondria was assayed by the luciferin-luciferase assay (Stanley & Williams, 1969) by using 100pmol of ATP standard made up in the corresponding mitochondrial incubation medium with HClO$_4$. Control experiments showed that standard curves were linear over the range 10–200pmol.

NADH and NAD$^+$ (after conversion into NADH) were assayed with NADH luciferase (Stanley, 1971). This enzyme will also utilize NADPH. For the determination of the d.p.m. due to NADPH, and hence of NADH by difference, each sample was reassayed after incubation for 5min in the luciferase assay buffer containing 1mM-pyruvate and 1.5 units of lactate dehydrogenase to remove NADH (at room temperature). Control experiments showed that the conversion of NADH into NAD$^+$ was complete within 1–2min. Standard NADH solutions were taken through the entire procedure, i.e. 0.5 and 1nmol of NADH was added to 0.5ml of KCl medium and 0.6vol. of ethanolic KOH was added and $10\mu l$ was then added in the luciferase assay. For the assay of NAD$^+$, $100\mu l$ of HClO$_4$ extract of mitochondria (or of NAD$^+$ standards, 5 or 10nmol in 0.5ml of KCl medium plus $10\mu l$ of HClO$_4$) was added to 0.9ml of 75 mM-semicarbazide/200mM-glycine/150mM-ethanol, pH10, and 2.5units of yeast ethanol dehydrogenase (EC 1.1.1.1) was added. After 5min, the NADH formed was assayed with luciferase. Control experiments showed that the conversion of NAD$^+$ into NADH is complete within 1–2min [the conditions of conversion were those described by Estabrook & Maitra (1962)]. Control experiments showed that NADH in ethanolic KOH or NAD$^+$ in HClO$_4$ were stable for at least 4h; assays were completed well within this time. The sensitivity of the luciferase assay was such as to detect 2pmol of NADH and assays were linear in the range relevant to these assays (2–20pmol).

CoA was assayed as NADH formed in the 2-oxo-glutarate dehydrogenase reaction with NADH/luciferase. Acetyl-CoA was assayed as CoA released by citrate synthase and oxaloacetate. The procedure was as follows. An $HClO_4$ extract of mitochondria (0.4 ml) was mixed with 2 ml of buffer (0.12 M-KCl containing 0.1 M-Tris/HCl/2 mM-$MgCl_2$/5 mM-2-mer-captoethanol, pH 7.8). For the assay of CoA, 0.5 ml of this mixture of $HClO_4$ extract plus buffer was in-cubated at 30°C for 4 min with NAD^+ (to 0.21 mM), thiamin pyrophosphate (to 0.78 mM), 2-oxoglutarate (to 2.5 mM) and 20 munits of 2-oxoglutarate dehydro-genase. The reaction was complete within 4 min and at the end of the incubation the tubes were cooled to 0°C. For the assay of (acetyl-CoA + CoA) a parallel incubation mixture contained additionally oxalo-acetate (to 0.25 mM) and citrate synthase (7 munits). The NADH produced was assayed by the NADH/luciferase assay (Stanley, 1971), by using an NADH standard (100 pmol). With suitable amounts of luci-ferase the standard curves for NADH were linear up to at least 200 pmol of NADH. CoA standards (3 nmol) were taken through the entire procedure in KCl medium. Acetyl-CoA standards (6 nmol) were also included. The recoveries of CoA and of acetyl-CoA were approx. 90%. Corrections for this re-covery were applied in calculating values for mito-chondrial CoA and for acetyl-CoA. In assaying mito-chondrial extracts, the volume added in the luciferase assay contained 10–200 pmol of NADH, depending on the sensitivity of a particular batch of luciferase.

Measurement of intramitochondrial dichloroacetate concentration. The concentration of dichloroacetate in the mitochondrial-matrix water was calculated as follows. In one set of incubations with ^3H_2O and [U-^{14}C]sucrose dual isotope counting of the mito-chondrial extract and of the incubation medium (see above) gave mitochondrial spaces for ^3H_2O and [U-^{14}C]sucrose. In parallel incubations with [U-^{14}C]-sucrose and dichloro[2-^3H]acetate, dual isotope counting of mitochondrial extracts and of the incuba-tion medium gave spaces for [U-^{14}C]sucrose and dichloro[2-^3H]acetate. These spaces were calculated from the general formula:

$$\text{Space } (\mu l/\text{mg of mitochondrial protein}) = \frac{\text{d.p.m./mg in mitochondrial pellet}}{\text{d.p.m./}\mu l \text{ in incubation medium}}$$

By using sucrose as an extramitochondrial marker the mitochondrial matrix spaces for dichloroacetate and water were calculated from (dichloroacetate space−sucrose space) and (water space−sucrose space) respectively. The concentration of dichloro-acetate in the mitochondrial matrix water (μM) was calculated from (matrix dichloroacetate space in $\mu l/\text{mg}$)×(medium dichloroacetate concentration in pmol/μl)×(matrix water space in $\mu l/\text{mg}$)$^{-1}$.

Dual isotope counting was done by liquid-scintillation spectrometry and quench corrections were obtained by the use of an external standard. Corrections were applied for quenching by mito-chondrial proteins and by lipids by assays of the radioactivity in the incubation media in the presence of extracts of mitochondria incubated without radio-active compounds. These corrections were small (less than 5%).

Units of enzyme activity. A unit of enzyme activity catalyses the disappearance of substrate or the appearance of product at a rate of 1 μmol/min at the temperature quoted in the Tables or text or Figures. For pyruvate dehydrogenase phosphate phosphatase units were calculated in terms of μmol of P_i released/min. For pyruvate dehydrogenase kinase, activities are given as pmol of protein-bound phosphate formed/unit of pyruvate dehydrogenase in the time shown.

Results and Discussion

Pyruvate dehydrogenase activity in perfused rat heart; effects of alloxan-diabetes, acetate, n-octanoate, palmitate, pyruvate and dichloroacetate

Control experiments validating the methods used for the extraction and the assay of pyruvate dehydro-genase in rat heart have been described (Whitehouse & Randle, 1973; Whitehouse *et al.*, 1974). The only variation in technique in the present experiments was the inclusion of 5 mM-2-mercaptoethanol in the extraction medium.

As Table 1 shows, the pyruvate dehydrogenase activity of hearts from fed non-diabetic rats perfused with glucose (5.5 mM) and insulin (20 munits/ml) averaged 5.5 units/g dry wt. (approx. 24% of the total activity, which is the sum of active and inactive forms). These values agree well with those of a previous study [5.6 units/g dry wt. and 20% of total (Whitehouse *et al.*, 1974)]. Alloxan-diabetes decreased the pro-portion of active dehydrogenase substantially, regardless of whether the activities were calculated on a dry-weight basis, as percentage of total activity (which was not changed by diabetes) or per unit of citrate synthase (another mitochondrial enzyme). Perfusions with acetate, *n*-octanoate or palmitate decreased the proportion of active dehydrogenase in hearts of non-diabetic rats, but the effects were perhaps less marked than those of diabetes. The effects of diabetes, acetate or palmitate are qualita-tively similar to those described by Wieland *et al.* (1971*a*,*b*).

Pyruvate (5 or 25 mM) or dichloroacetate (0.1 or 1 mM) markedly increased the proportion of active dehydrogenase in hearts of fed non-diabetic animals (Table 1). Presumably inhibition of pyruvate de-hydrogenase kinase by pyruvate (Linn *et al.*, 1969*a*,*b*) or dichloroacetate (Whitehouse *et al.*, 1974) facilitates

Table 1. *Pyruvate dehydrogenase activity in perfused rat heart; effects of alloxan-diabetes, pyruvate, dichloroacetate, acetate, octanoate and palmitate*

Hearts were perfused for 10 min at 38°C with bicarbonate-buffered saline medium (Krebs & Henseleit, 1932) gassed with O_2+CO_2 (95:5) and additions as shown. At the end of perfusion the tissue was frozen with a tissue clamp cooled in liquid N_2, powdered, extracted and assayed for pyruvate dehydrogenase as described in the Experimental section. Concentrations: glucose, 5.5 mM; insulin, 20 munits/ml; acetate, 5 mM; albumin, 20 g/l; palmitate albumin, 0.6 mM; octanoate, 1 mM. Results are means ± S.E.M. for the numbers of hearts shown in parentheses.

	Additions to perfusion medium			Activity of pyruvate dehydrogenase (active form)		
Rats	Pyruvate (mM)	Dichloro-acetate (mM)	Other	(units/g dry wt.)	(% of total)	(munits/unit of citrate synthase)
Non-diabetic	—	—	Glucose+insulin	6.72±0.73 (4)	28.6 ±1.8 (4)	9.5 ±0.72 (4)
	5	—		19.2 ±1.2 (4)	68.9 ±1.4 (4)	32.0 ±1.79 (4)
	25	—		23.6 ±0.29 (4)	77.6 ±2.4 (4)	38.6 ±1.23 (4)
Diabetic	—	—	Glucose+insulin	0.32±0.005 (8)	1.53±0.003 (8)	0.71±0.09 (8)
	5	—		2.66±0.61 (8)	13.2 ±3.0 (8)	3.98±0.78 (8)
	25	—		4.66±0.81 (8)	23.1 ±4.0 (8)	8.38±1.74 (8)
Non-diabetic	—	—	Glucose+insulin	6.65±0.31 (4)	25.6 ±2.78 (4)	9.5 ±0.65 (4)
	—	1		20.6 ±0.62 (6)	94.9 ±0.85 (6)	29.4 ±0.88 (6)
Diabetic	—	—	Glucose+insulin	0.31±0.005 (8)	1.53±0.03 (8)	0.71±0.09 (8)
	—	1		11.6 ±1.90 (6)	35.4 ±4.69 (6)	16.55±2.71 (6)
Non-diabetic	—	—	Glucose+insulin+ albumin	4.6 ±0.7 (3)	—	—
	5	—		22.7 ±0.89 (4)	—	—
	—	—	Glucose+insulin+ albumin+palmitate	1.0 ±0.21 (4)	—	—
	5	—		17.7 ±0.61 (4)	—	—
Non-diabetic	—	—	Glucose+insulin	3.33±0.42 (6)	18.9 ±0.9 (4)	—
	5	—		13.11±1.16 (6)	46.9 ±1.12 (4)	—
	—	—	Glucose+insulin+ acetate	1.41±0.17 (6)	7.0 ±0.75 (6)	—
	5	—		11.51±1.09 (6)	41.7 ±3.23 (4)	—
Non-diabetic	—	—	Glucose+insulin	5.8 ±1.4 (2)	—	—
	—	0.1		11.6 ±0.04 (2)	—	—
	—	1		12.1 ±0.7 (2)	—	—
	—	—	Glucose+insulin+ acetate	0.35±0.3 (2)	—	—
	—	0.1		1.38±0.15 (2)	—	—
	—	1		8.9 ±0.4 (2)	—	—
Non-diabetic	—	—	Glucose+insulin	5.8 ±1.4 (2)	—	—
	5	—		10.7 ±0.58 (2)	—	—
	25	—		10.8 ±3.4 (2)	—	—
	—	—	Glucose+insulin+ octanoate	1.1 ±0.4 (2)	—	—
	5	—		2.6 ±0.24 (2)	—	—
	25	—		15.3 ±1.3 (2)	—	—

the conversion of the inactive form into the active form by action of the phosphatase. Pyruvate and dichloroacetate had similar effects in hearts of diabetic animals, but the proportion of active dehydrogenase was markedly lower. Effects that were qualitatively similar but perhaps smaller than those of diabetes, were seen in hearts of non-diabetic animals on perfusion with acetate, n-octanoate or palmitate (significant effects for acetate ± 0.1 mM-dichloroacetate; n-octanoate ± 5 mM-pyruvate; palmitate ± 5 mM-pyruvate; Table 1).

The effects of diabetes, acetate, octanoate or palmitate suggest either that the activity of pyruvate dehydrogenase kinase is increased or that the activity of pyruvate dehydrogenase phosphate phosphatase is decreased by these agents, or both. Such changes could be the result of an alteration in the concentrations of kinase or of phosphatase resulting possibly from altered rates of synthesis or degradation, or from an activity change through covalent modification, or from an activity change through alteration in the concentration of effectors of the kinase or phosphatase reactions. Evidence bearing on these possibilities is considered in the sections that follow.

Pyruvate dehydrogenase activity in mitochondria from hearts of non-diabetic and alloxan-diabetic rats; effects of pyruvate and of dichloroacetate

Pyruvate dehydrogenase was assayed spectro-photometrically in extracts of mitochondria by coupling to arylamine acetyltransferase. Oxalo-acetate can interfere in this assay by enabling citrate synthase (present in mitochondrial extracts) to compete with arylamine acetyltransferase for the acetyl-CoA generated by the pyruvate dehydrogenase reaction. Oxaloacetate is formed from many of the substrates utilized by mitochondria (e.g. malate, succinate, 2-oxoglutarate, glutamate). This difficulty has been overcome by separating a mitochondrial pellet by centrifugation (20s), removing the incubation medium by aspiration and then freezing the mitochondrial pellet (<10s). The small quantities of metabolites present in the mitochondrial extract under these conditions do not interfere with the assay of pyruvate dehydrogenase by coupling to arylamine acetyltransferase (shown by control experiments using acetyl-CoA as substrate). The frozen mitochondrial pellet was then extracted by ultrasonic disintegration into extraction medium at −10°C (with glycerol as antifreeze). This technique, which was based on that described by Walajtys *et al.* (1974), is more convenient than that previously used in this laboratory (see Martin *et al.*, 1972), but gave very similar results. Centrifugation of mitochondria for periods from 5 to 30s after 5min of incubation with 0.5mM-L-malate plus 5mM-2-oxoglutarate gave similar values for active and for total pyruvate dehydrogenase; 20s was chosen for convenience. Centrifuging for more than 30s increased the pyruvate dehydrogenase activity (active form) of extracts of mitochondria incubated with 2-oxoglutarate plus malate. Attempts to assay pyruvate dehydrogenase in extracts prepared from mitochondria without centrifugation were not satisfactory. Extracts were prepared (after the addition of EDTA to 5mM) by freezing and thawing three times or by ultrasonic disintegration or with the Polytron homogenizer. Pyruvate dehydrogenase was assayed by the formation of $^{14}CO_2$ from [1-^{14}C]pyruvate. These techniques gave lower values for both active dehydrogenase and the total dehydrogenase than the centrifuge method, with mitochondria incubated for 5min without substrate or with 2-oxoglutarate plus malate. Taylor *et al.* (1975) in experiments with liver mitochondria have concluded that the extraction and assay of pyruvate dehydrogenase without centrifugation is superior to methods involving centrifugation. We have no evidence to support this conclusion in our experiments with heart mitochondria and the experiments of Taylor *et al.* (1975) did not include the estimations of total pyruvate dehydrogenase after incubation with added phosphatase. The method used here relied on the presence of EDTA, dilution and low temperature, to inhibit phosphatase and kinase activities during extraction and assay. The inclusion of fluoride (an inhibitor of the phosphatase) or of pyruvate or of dichloroacetate (inhibitors of the kinase) did not modify the results obtained. The only disadvantage of the glycerol extraction medium was that the extracts were not suitable for the assay of total pyruvate dehydrogenase. Incubation with phosphatase gave much less pyruvate dehydrogenase activity than in extracts prepared with phosphate medium (see the Experimental section). It was found, however, that incubation of mitochondria for 5min in the absence of substrate led to the conversion of at least 90% of the dehydrogenase into the active form. It has been convenient to use the activity of pyruvate dehydrogenase in mitochondria incubated without substrate as an estimate of the total pyruvate dehydrogenase activity in any given mitochondrial preparation.

As shown in Table 2 the pyruvate dehydrogenase activity of mitochondria from hearts of non-diabetic rats incubated for 5min without substrate was 75.4munits/mg of mitochondrial protein. The corresponding value for diabetic rats was 73.3. These values correspond closely to the total pyruvate dehydrogenase activity of rat heart mitochondria. Incubation with respiratory substrates (oxoglutarate plus malate) increased the ATP concentration and diminished the proportion of active dehydrogenase. The rise in ATP was somewhat smaller in mitochondria from hearts of diabetic rats than in the non-diabetic controls, whereas the pyruvate dehydrogenase activity fell to a lower value. This difference in pyruvate dehydrogenase activity is qualitatively similar to but quantitatively smaller than the difference observed in the perfused heart (see Table 1).

In mitochondria from hearts of non-diabetic rats incubated with oxoglutarate plus malate, pyruvate or dichloroacetate increased the proportion of active dehydrogenase, presumably as a result of the inhibition of pyruvate dehydrogenase kinase (Cooper *et al.*, 1974; Whitehouse *et al.*, 1974). As shown in Table 2 the threshold concentration of pyruvate has varied in different experiments from 50 to 250μM. As shown in Table 2 and Fig. 1, pyruvate at low concentrations (with malate) functions as a respiratory substrate and increases ATP and lowers the proportion of active dehydrogenase. At concentrations up to 50–100μM, pyruvate plus malate is as effective in lowering pyruvate dehydrogenase activity as is oxoglutarate plus malate. With pyruvate plus malate, increasing the concentration of pyruvate above 50–100μM increases the proportion of active dehydrogenase. As is also shown in Table 2, increasing the concentration of pyruvate (in the presence of malate) from 10 to 25 or 50μM decreased the activity of pyruvate dehydrogenase without further changing the ATP concentration. This is one of a number of

Table 2. *Pyruvate dehydrogenase activity of mitochondria prepared from hearts of normal and alloxan-diabetic rats*

Mitochondria (0.5 or 1 mg) were incubated for the time shown at 30°C in 0.5 or 1 ml of KCl medium containing (except in the no-substrate controls and Expt. 3) 5mM-2-oxoglutarate and 0.5mM-L-malate and the concentrations of pyruvate or dichloroacetate as shown. The time of incubation, volumes and mitochondrial concentrations were such that no more than 20% of substrate was utilized. For details of preparation of mitochondria, extraction and assay of enzymes and ATP see the Experimental section. Where given, standard errors were computed from values obtained with different preparations of mitochondria, the numbers of which are given in parentheses. Each preparation was made from at least four hearts and incubations with any one batch were made in triplicate. The total activity of pyruvate dehydrogenase was approximately the same as the value for the no-substrate control. The concentrations of citrate synthase were 1.20 (non-diabetics) and 1.23 (diabetics) units/mg of mitochondrial protein.

Expt.	Incubation		Time (min)	Pyruvate dehydrogenase activity (munits/mg of mitochondrial protein)		ATP concn. (nmol/mg of mitochondrial protein)	
				Non-diabetic	Diabetic	Non-diabetic	Diabetic
1	No-substate control		5	75.4±5.0 (13)	73.3±7.3 (6)	0.94±0.14 (12)	1.02±0.12 (7)
	5mM-2-oxoglutarate+ 0.5mM-L-malate with						
	Pyruvate (mM)	Dichloro-acetate (mM)		Pyruvate dehydrogenase activity (% of no-substrate control)			
				Non-diabetic	Diabetic		
	—	—	5	19.2±1.5 (13)	14.4±1.8 (6)*	4.25±0.39 (12)	3.62±0.38 (7)
	0.1	—	5	15.9	12.1	—	—
	0.25	—	5	34.4±2.7 (7)	19.4±4.5 (3)	—	—
	0.40	—	5	58.4	18.5	—	—
	0.50	—	5	77.6±2.2 (13)	26.0±2.9 (6)**	4.20±0.83 (2)	2.80±0.25 (2)
	2	—	5	82.2±3.1 (3)	48.9±6.3 (3)†	—	—
	10	—	5	109.8	54.3	—	—
	—	4	5	77.9	24.1	—	—
	—	10	5	91.6	32.0	—	—
	—	50	5	94.6	68.9	—	—
2	—	—	5	10.6±0.34 (16)	—	—	—
	0.05	—	5	15.1±0.17 (16)	—	—	—
	No-substrate control		5	65.8±2.8 (8)			
3	0.5mM-L-malate with						
	Pyruvate (mM)	Dichloro-acetate (mM)					
	0.01	—	1	18.1±0.43 (4)	—	2.1 ±0.04 (2)	—
	0.025	—	1	9.9 ±0.87 (4)	—	2.2 ±0.04 (2)	—
	0.05	—	1	9.6±0.81 (4)	—	2.0 ±0.03 (2)	—
	0.10	—	1	25.5±1.63 (4)	—	2.1 ±0.06 (2)	—
	No-substrate control		1	48.1±7.2 (4)	—	2.3 ±0.14 (2)	—
	No-substrate control		5	73.1±2.8 (4)	—	0.7 ±0.02 (2)	—

* $P < 0.02$ (paired difference) against non-diabetic control.
** $P < 0.001$.
† $P < 0.05$, against non-diabetic control.
Others $P > 0.05$.

discrepancies between ATP concentration and dehydrogenase activity noted in the present study and it is discussed in more detail in later sections. With dichloroacetate substantial activation of the dehydrogenase was seen at 4μM and activation was largely complete at 10 or 50μM, in agreement with previous findings (Whitehouse *et al.*, 1974).

The ability of pyruvate or of dichloroacetate to effect the conversion of pyruvate dehydrogenase into its active form was markedly decreased in mitochondria from hearts of alloxan-diabetic rats. This difference between mitochondria from hearts of diabetic and non-diabetic rats was evident at all concentrations of pyruvate from 250μM to 10mM
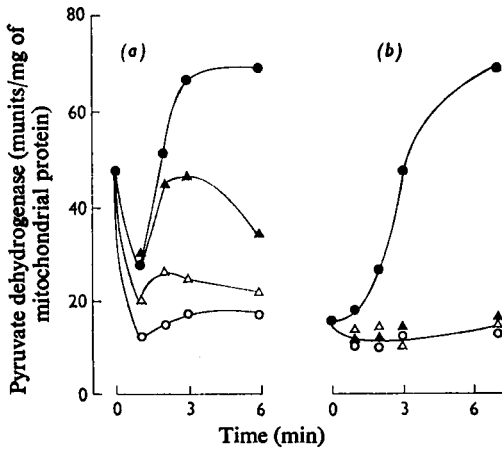
Fig. 1. *Activity of pyruvate dehydrogenase (active form) in heart mitochondria from non-diabetic (a) or diabetic (b) rats*

Rat heart mitochondria (0.5 mg of mitochondrial protein) were incubated for the time shown at 30°C in KCl medium containing: ●, no substrate added; ○, 0.5 mM-L-malate plus 0.1 mM-pyruvate; △, 0.5 mM-L-malate plus 0.25 mM-pyruvate; ▲, 0.5 mM-L-malate plus 0.5 mM-pyruvate. For details of extraction and assay of pyruvate dehydrogenase see the Experimental section.

and at 4, 10 or 50 μM-dichloroacetate, although statistical significance was only established at 0.5 and 2 mM-pyruvate. The difference was evident with activities of pyruvate dehydrogenase (active form) expressed per mg of mitochondrial protein or per unit of citrate synthase or as a percentage of total pyruvate dehydrogenase activity. This difference in responsiveness to pyruvate is shown in a different way in Fig. 1, which shows the time-course of pyruvate dehydrogenase activity (active form) in rat heart mitochondria incubated without substrate or with 0.5 mM-malate plus pyruvate (concns. 0.1, 0.25 or 0.5 mM). In mitochondria from non-diabetic rats the lowest concentration of pyruvate (0.1 mM) increased ATP (not shown) and lowered pyruvate dehydrogenase activity to approx. 20% of that seen in the absence of substrate. With higher concentrations of pyruvate (0.25 or 0.5 mM) smaller diminutions in pyruvate dehydrogenase activity were seen, presumably as a result of the inhibition of pyruvate dehydrogenase kinase by pyruvate. In mitochondria from hearts of diabetic rats all three concentrations of pyruvate were associated with a low proportion of active dehydrogenase (approx. 16% of the no-substrate value). In further experiments with the oxygen electrode it was found that the maximum rate of O$_2$ consumption with 1 μmol of ADP (concn.

Table 3. *Effect of alloxan-diabetes on the activity of pyruvate dehydrogenase phosphate phosphatase in rat heart and in rat heart mitochondria*

Hearts were removed under Nembutal anaesthesia and frozen with a tissue clamp cooled in liquid N$_2$. The frozen tissue was powdered and 10 mg of powder extracted in 20 mM-potassium phosphate, 5 mM-2-mercaptoethanol, pH 7.0, with a Polytron PT 10 homogenizer (20 s, position 4). After centrifuging for 2.5 min in an Eppendorf 3200 centrifuge, the activity of pyruvate dehydrogenase phosphate phosphatase was assayed on 10 μl of supernatant in the presence of 10 mM-EGTA or of 10 mM-EGTA+9.75 mM-CaCl$_2$+25 mM-MgCl$_2$ as described by Randle *et al.* (1974). Mitochondria were prepared and phosphatase was extracted (without incubation of mitochondria) and assayed as described in the Experimental section. Results are means ± S.E.M. for the numbers of observations in parentheses.

	Pyruvate dehydrogenase phosphate phosphatase		
Assay in presence of Rat heart	(munits/g dry wt. of heart)	(μunits/unit of citrate synthase)	(munits/unit of pyruvate dehydrogenase)
Non-diabetic			
EGTA	17.0 ± 1.58 (5)	35 ± 0.25 (5)	0.65
Ca^{2+}+EGTA	46.1 ± 1.59 (5)	110 ± 9 (5)	1.76
Diabetic			
EGTA	13.2 ± 0.48 (5)	33 ± 2.2 (5)	0.65
Ca^{2+}+EGTA	40.6 ± 2.27 (5)	100 ± 10 (5)	2.00
Rat heart mitochondria			
Non-diabetic			
EGTA	—	25 ± 4.9 (4)	0.45
Ca^{2+}+EGTA	—	62 ± 10.2 (4)	1.06
Diabetic			
EGTA	—	17 ± 1.4 (4)	0.35
Ca^{2+}+EGTA	—	56 ± 3.5 (4)	1.03

0.25 mM), 0.5 mM-L-malate and 0.5 mM-pyruvate was approx. 30% lower in mitochondria from diabetic hearts as compared with non-diabetic controls. With 5 mM-2-oxoglutarate and 0.5 mM-L-malate as substrates the rates of O_2 consumption were identical.

These results suggest that the differences in pyruvate dehydrogenase activity in the perfused hearts of non-diabetic rats persist in mitochondria prepared from hearts of these animals. Apparently the mitochondria are imprinted in some way such that the characteristic effects of pyruvate or of dichloroacetate in increasing pyruvate dehydrogenase activity are impaired in mitochondria from hearts of diabetic animals.

As shown in Table 3 this difference between hearts of non-diabetic or diabetic rats or of mitochondria prepared from them is not due to any difference in the activity of pyruvate dehydrogenase phosphate phosphatase extracted from the heart or from heart mitochondria. The maximum phosphatase activity of the heart (measured with Ca^{2+}/EGTA buffer; 9.75 mM-CaCl$_2$/10 mM-EGTA; free Ca^{2+}, 10 μM) was approx. 40 munits/g dry wt. or 110 μunits/unit of citrate synthase or 1.8 munit/unit of pyruvate dehydrogenase. There was no significant difference between the hearts of non-diabetic or diabetic rats. The activity of the phosphatase in extracts of rat heart mitochondria (per unit of citrate synthase or per unit of pyruvate dehydrogenase) was approximately two-thirds of that in the heart, but no difference was detected as between non-diabetic or diabetic animals. This might suggest that extramitochondrial phosphatase(s) can also hydrolyse pyruvate dehydrogenase phosphate. More convincing evidence for this supposition has come from similar experiments with rat epididymal adipose tissue, where the difference is much larger (Stansbie et al., 1976).

It should be emphasized that these experiments merely show that diabetes does not lower the activity of pyruvate dehydrogenase phosphate phosphatase under the conditions of extraction used and with pig heart pyruvate dehydrogenase phosphate as substrate. These experiments do not rule out the possibility of inhibition of the phosphatase reaction in diabetic mitochondria by modifications of the concentration of some inhibitor or activator or other modification which is lost during extraction.

We have also considered the possibility that pyruvate or dichloroacetate may be concentrated to a lesser extent by mitochondria of diabetic hearts than by their non-diabetic controls. This is difficult to test with pyruvate, since pyruvate is rapidly decarboxylated by rat heart mitochondria (see Table 5). We have, however, studied the accumulation of dichloro-[2-³H]acetate by mitochondria from hearts of diabetic and non-diabetic rats and these results are shown in Table 4. Dichloroacetate and pyruvate share the same carrier (Halestrap, 1975). In mitochondria from hearts of non-diabetic rats dichloroacetate was concentrated 16-fold at an external concentration of 10 μM and tenfold at an external concentration of 50 μM. This finding may explain the apparently greater sensitivity to external dichloroacetate of the pyruvate dehydrogenase kinase reaction in mitochondria as compared with the extracted dehydrogenase complex (Whitehouse et al., 1974).

The matrix water space of mitochondria from hearts of diabetic rats was approximately twice that of the non-diabetic controls. Mitochondria from hearts of diabetic rats concentrated dichloroacetate about half as effectively as did mitochondria from non-diabetic hearts. Thus differences in the accumulation could be a factor in the diminished effects of dichloro-

Table 4. *Effect of alloxan-diabetes on the matrix water space and on the distribution of dichloroacetate across the inner membrane of rat heart mitochondria*

Rat heart mitochondria (1–2 mg) were incubated at 30°C for 5 min in 0.5 ml of KCl medium (see the Experimental section) containing 5 mM-2-oxoglutarate, 0.5 mM-L-malate and either 2.5 μCi of ³H$_2$O plus 0.25 μCi of [U-¹⁴C]sucrose or 25 nCi of [U-¹⁴C]sucrose plus 0.25 μCi of dichloro[2-³H]acetate. Mitochondria and supernatant were separated by centrifugation and assayed for radioactivity and spaces calculated as given in the Experimental section. Results are given as means ± S.E.M. for the numbers of observations in parentheses.

	Space (μl/mg of protein) or concn. (μM) in heart mitochondria from	
	Non-diabetic rats	Alloxan-diabetic rats
Water space (mitochondrial matrix)	0.296 ± 0.035 (19)	0.537 ± 0.061 (12)
Dichloroacetate space		
(10 μM in medium)	4.76 ± 0.24 (15)	5.02 ± 0.18 (8)
(50 μM in medium)	2.99 ± 0.19 (16)	2.70 ± 0.17 (12)
Dichloroacetate concn.		
(10 μM in medium)	161 ± 8.2 (15)	93.5 ± 3.4 (8)*
(50 μM in medium)	505 ± 32 (16)	251.0 ± 15.6 (12)*

*$P < 0.001$ against non-diabetic control.

acetate or of pyruvate on dehydrogenase activity in diabetic hearts or their mitochondria. However, it seems unlikely that this is the only factor involved. The concentration of dichloroacetate in mitochondria from hearts of diabetic rats with 50μM-dichloroacetate in the medium was 250μM; this is 50% greater than the concentration in non-diabetic mitochondria at a concentration in the medium of 10μM (Table 4). The activity of pyruvate dehydrogenase in diabetic mitochondria was, however, lower than that of non-diabetic mitochondria at these external concentrations. The diminished accumulation of dichloroacetate by diabetic mitochondria may be due to some degree of uncoupling. The respiratory-control ratio of mitochondria was lower than that of non-diabetic mitochondria and the ATP concentrations during incubation with oxoglutarate plus malate were consistently lower.

Effect of palmitoyl-L-carnitine, acetyl-L-carnitine and 2-oxoglutarate on the rate of decarboxylation of [1-^{14}C]pyruvate by rat heart mitochondria

The oxidation of fatty acids and of ketone bodies in perfused rat heart inhibits the oxidation of pyruvate formed from glucose (Garland *et al.*, 1964). This effect of fatty acids and of ketone bodies has been attributed to the substantial increase in the ratio [acetyl-CoA]/[CoA], which they induce and which inhibits pyruvate dehydrogenase in the presence of NADH (Garland & Randle, 1964*a,b*). This effect of fatty acids and of ketone bodies is also associated with a diminution in the proportion of active (dephosphorylated) pyruvate dehydrogenase in the perfused heart (Wieland *et al.*, 1971*b*). In order to define the

relationship between end-product inhibition of pyruvate dehydrogenase by acetyl-CoA (and/or NADH) and inactivation of pyruvate dehydrogenase by phosphorylation, palmitoyl-L-carnitine and acetyl-L-carnitine have been used to inhibit pyruvate oxidation in rat heart mitochondria (Bremer, 1966).

Pyruvate oxidation has been assayed in mitochondria by measurement of the rate of decarboxylation of [1-^{14}C]pyruvate. The conditions were arranged so that no more than 20% of the pyruvate in the medium was utilized during a 5 min period of incubation. The rate of decarboxylation was constant under these conditions and was proportional to the concentration of mitochondria. The concentrations of pyruvate used were 50 and 500μM, so that measurements could be made at different activities of pyruvate dehydrogenase (Table 2). These results are shown in Table 5.

The rate of pyruvate oxidation by rat heart mitochondria was 6.85 nmol/min per mg of protein with 50μM-pyruvate plus 0.5 mM-L-malate. The activity of pyruvate dehydrogenase (active form) was 15.1 munits/mg of protein. The rate of pyruvate oxidation was thus approx. 46% of the $V_{max.}$ of the pyruvate dehydrogenase activity. This seems reasonable, because the presence of acetyl-CoA and NADH in mitochondria may have led to some degree of end-product inhibition. With 0.5 mM-pyruvate the rate of oxidation of pyruvate was increased (by approx. 74%) and the activity of pyruvate dehydrogenase was increased by approx. 150%.

Palmitoyl-L-carnitine at concentrations between 6.25 and 50μM leads to increasing inhibition of pyruvate oxidation, amounting to approx. 90% at the

Table 5. *Effect of palmitoyl-L-carnitine, acetyl-L-carnitine and 2-oxoglutarate on the decarboxylation of pyruvate by rat heart mitochondria*

Decarboxylation of pyruvate was assayed by the formation of $^{14}CO_2$ from [1-^{14}C]pyruvate on incubation of rat heart mitochondria (1 mg of mitochondrial protein) for 5 min at 30°C in 4 ml of KCl medium containing 0.5 mM-L-malate with [1-^{14}C]-pyruvate (1-2μCi) at the concentration shown and with other additions as given. Activity of pyruvate dehydrogenase was assayed in parallel incubations in Eppendorf tubes of 0.5 mg of mitochondrial protein in 1 ml of KCl medium. For details of assay see the Experimental section. Results are means ± S.E.M. for the numbers of observations in parentheses.

Pyruvate concn. (μM)	Other additions to incubation (in addition to 0.5 mM-L-malate)	Pyruvate decarboxylation (nmol/min per mg of mitochondrial protein)	Pyruvate dehydrogenase activity (munits/mg of mitochondrial protein)
50	None	6.85 ± 0.17 (18)	15.1 ± 0 (2)
50	6.25 μM-palmitoylcarnitine	5.48 ± 0.32 (3)	—
50	12.5 μM-palmitoylcarnitine	2.52 ± 0.014 (3)	—
50	25 μM-palmitoylcarnitine	1.54 ± 0.057 (3)	—
50	50 μM-palmitoylcarnitine	0.82 ± 0.027 (18)	14.2 ± 0.64 (7)
50	0.5 mM-acetylcarnitine	2.52 ± 0.06 (6)	14.2 ± 0.62 (13)
50	5 mM-2-oxoglutarate	4.05 ± 0.41 (3)	17.1 ± 1.35 (13)
500	None	11.8 ± 0.24 (4)	37.6 ± 1.25 (8)
500	50 μM-palmitoylcarnitine	5.2 ± 0.04 (4)	39.0 ± 12.3 (6)
No-substrate control		—	67.7 ± 0.41 (4)

highest concentration. The activity of extracted pyruvate dehydrogenase (active form) with 50μM-pyruvate plus 0.5mM-L-malate was unaffected by 50μM-palmitoyl-L-carnitine notwithstanding the marked difference in the rate of pyruvate oxidation. Essentially similar results were obtained in the absence of L-malate (not shown). Apparently end-product inhibition of pyruvate dehydrogenase was solely responsible for the inhibition of pyruvate oxidation by palmitoyl-L-carnitine in these experiments.

To measure the mitochondrial concentrations of acetyl-CoA and CoA it was necessary to increase the concentration of mitochondria to 4mg of mito-chondrial protein/ml and to shorten the incubation time to 45s. In mitochondria incubated with 50μM-pyruvate plus 0.5mM-L-malate the concentrations of acetyl-CoA and CoA were 0.12±0.08 and 1.3± 0.11 nmol/mg of protein respectively for four obser-vations. With addition of palmitoyl-L-carnitine (50μM) the concentration of acetyl-CoA increased to 0.6±0.09 nmol/mg of protein and that of CoA fell to 0.41±0.06 nmol/mg of protein (four observations). Palmitoyl-L-carnitine thus increased the ratio [acetyl-CoA]/[CoA] from 0.092 to 1.45. The mitochondrial concentration of ATP was not changed by palmitoyl-carnitine (results not shown).

Palmitoylcarnitine also inhibited pyruvate oxida-tion without alteration in the activity of pyruvate dehydrogenase (active form) at 0.5mM-pyruvate (Table 5). Acetyl-L-carnitine (0.5mM) also inhibited pyruvate oxidation (50μM-pyruvate) without chang-ing the activity of extracted pyruvate dehydrogenase (Table 5) or the mitochondrial concentration of ATP (results not shown). Higher concentrations of acetyl-L-carnitine (5mM) produced a greater degree of inhibition of pyruvate oxidation, but the concen-tration of ATP fell and the activity of pyruvate dehydrogenase increased (results not shown). 2-Oxo-glutarate, which through the 2-oxoglutarate dehydro-genase reaction may compete with pyruvate dehydro-genase for CoA without forming acetyl-CoA, produced only a small inhibition of pyruvate oxida-tion.

These observations would suggest that palmitoyl-L-carnitine and perhaps acetyl-L-carnitine may inhibit oxidation of pyruvate in isolated heart mitochondria through end-product inhibition of pyruvate dehydro-genase. The responsible factor may be a high [acetyl-CoA]/[CoA] ratio. This inhibition of the pyruvate dehydrogenase reaction in mitochondria was not associated with detectable inactivation of the enzyme by phosphorylation.

Effect of substrates for respiration on pyruvate dehydrogenase activity and ATP concentration in rat heart mitochondria

In the perfused rat heart, diabetes or the oxidation of fatty acids is associated with increased muscle concentrations of acetyl-CoA, acetyl-L-carnitine, citrate, isocitrate, glutamate and malate and decreased muscle concentrations of aspartate and CoA (Bow-man, 1966; Williamson, 1965; Randle et al., 1966, 1970). Diabetes and the metabolism of palmitate are also associated with increased muscle concentrations of palmitoyl-CoA and of palmitoyl-L-carnitine. The possibility that the effects of diabetes or of the respiration of fatty acids on the proportion of active pyruvate dehydrogenase may be mediated in per-fused rat heart by one of these metabolites has been investigated in isolated rat heart mitochondria. The effects of palmitoyl-L-carnitine and of acetyl-L-carnitine in the presence of pyruvate have been discussed in the preceding section. Other results are given in Table 6 and Fig. 2 or in the text below.

The O_2 consumption of rat heart mitochondria in the presence of ADP (0.25mM) was well supported by acetyl-L-carnitine (0.5–5mM) plus malate (0.5mM), by 2-oxoglutarate (5mM) plus malate, by L-glutamate alone or with malate, by succinate (5mM), by palmitoyl-L-carnitine (50μM) alone or with malate,

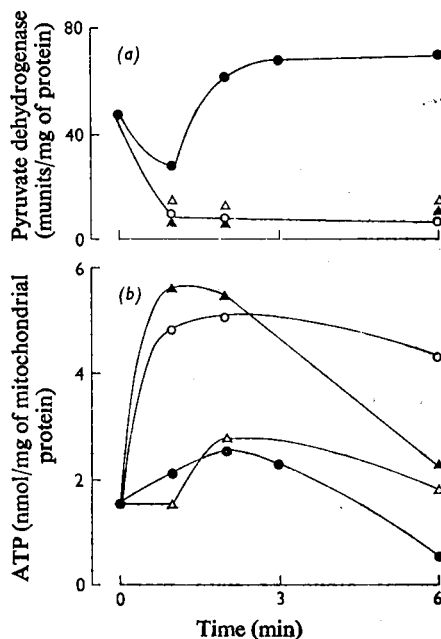

Fig. 2. *Activity of pyruvate dehydrogenase (active form) (a) and concentration of ATP (b) in rat heart mitochondria*

Rat heart mitochondria (1mg of mitochondrial protein) were incubated for the time shown at 30°C in KCl medium containing: ●, no substrate added; ○, 0.5mM-L-malate+ 5mM-2-oxoglutarate; △, 50μM-n-octanoate; ▲, 5mM-succinate.

Table 6. *Effect of substrates for respiration on the activity of pyruvate dehydrogenase in rat heart mitochondria*

Rat heart mitochondria (1 mg of mitochondrial protein) were incubated in 0.5–1 ml of KCl medium for 5 min at 30°C with additions as shown. In experiments with very low substrate concentrations the concentration of mitochondria was decreased to ensure that no more than 20% of the substrate was utilized during the period of incubation. For details of extraction and assay of pyruvate dehydrogenase and assay of ATP see the Experimental section. In each case the pyruvate dehydrogenase activity in the absence of substrate approximates closely to the total pyruvate dehydrogenase activity. The results are means ± S.E.M. for the numbers of observations in parentheses.

Additions to medium	Pyruvate dehydrogenase activity (munits/mg)	ATP concn. (nmol/mg)
No-substrate control	68.9 ± 5.5 (2)	1.3 ± 0.25 (2)
100 μM-succinate	64.5 ± 1.2 (3)	3.1 ± 0.04 (2)
250 μM-succinate	27.6 ± 2.1 (3)	5.3 ± 0.15 (2)
500 μM-succinate	12.2 ± 0.0 (3)	5.9 ± 0.30 (2)
1 mM-succinate	8.0 ± 0.78 (3)	5.9 ± 0.09 (2)
5 mM-succinate	5.7 ± 1.46 (3)	5.2 ± 0.65 (2)
No-substrate control	63.4 ± 3.3 (2)	0.58 ± 0.05 (2)
5 mM-2-oxoglutarate+0.5 mM-malate	13.3 ± 3.4 (2)	4.35 ± 0.10 (2)
5 mM-succinate	11.3 ± 0.7 (2)	2.18 ± 0.16 (2)
No-substrate control	68.7 ± 2.6 (5)	2.1 ± 0.03 (6)
50 μM-palmitoylcarnitine	12.2 ± 0.6 (5)	6.4 ± 0.13 (5)
20 μM-palmitoylcarnitine	36.3 ± 2.9 (5)	5.4 ± 0.03 (5)
10 μM-palmitoylcarnitine	59.7 ± 1.6 (6)	5.1 ± 0.08 (6)
5 mM-2-oxoglutarate+0.5 mM-malate	8.1 ± 0.8 (6)	6.3 ± 0.34 (6)
+50 μM-palmitoylcarnitine	10.1 ± 1.1 (6)	6.1 ± 0.08 (6)
+20 μM-palmitoylcarnitine	10.8 ± 1.7 (6)	6.2 ± 0.13 (6)
+10 μM-palmitoylcarnitine	8.0 ± 0.3 (6)	6.3 ± 0.06 (6)
No-substrate control	92.8 ± 2.8 (5)	1.2 ± 0.16 (5)
10 μM-*n*-octanoate	17.5 ± 0.32 (5)	1.9 ± 0.23 (5)
20 μM-*n*-octanoate	20.9 ± 0.50 (2)	1.5 ± 0.1 (2)
50 μM-*n*-octanoate	17.5 ± 0.92 (7)	1.7 ± 0.09 (5)
5 mM-2-oxoglutarate+0.5 mM-malate	15.2 ± 0.84 (5)	4.3 ± 0.27 (5)
No-substrate control	57.6 ± 4.2 (2)	0.9 ± 0.01 (3)
+oligomycin (10 μg/ml)	57.3 ± 0.41 (3)	0.7 ± 0.03 (3)
5 mM-2-oxoglutarate+0.5 mM-malate	7.2 ± 0.59 (3)	3.9 ± 0.10 (3)
+oligomycin (10 μg/ml)	6.3 ± 0.50 (3)	3.8 ± 0.11 (3)
50 μM-*n*-octanoate	9.4 ± 1.09 (3)	2.0 ± 0.02 (3)
+oligomycin (10 μg/ml)	41.8 ± 2.80 (3)	1.1 ± 0.04 (3)
0.2% albumin (no-substrate control)	80.7 ± 4.3 (2)	2.0 ± 0.04 (2)
+50 μM-palmitate	84.2 ± 17.3 (2)	2.5 ± 0.00 (2)
No-substrate control	49.5 ± 0.48 (3)	1.3 ± 0.70 (2)
5 mM-2-oxoglutarate+0.5 mM-L-malate	8.8 ± 0.55 (3)	4.8 ± 0.28 (2)
5 mM-L-glutamate+0.5 mM-L-malate	7.4 ± 0.47 (3)	4.6 ± 0.50 (2)

and by *n*-octanoate (50 μM). O_2 consumption was not supported by citrate alone or with malate, by malate alone, by acetate alone or with malate, or by palmitate (on albumin) alone or with malate. Succinate (0.5–5 mM), 2-oxoglutarate (5 mM) plus malate (0.5 mM), glutamate (5 mM) plus malate, palmitoyl-carnitine (50 μM) ± malate, and *n*-octanoate (10–50 μM) were equally effective in causing phosphorylation and inactivation of pyruvate dehydrogenase in isolated rat heart mitochondria (Table 6, or results not shown). With some exceptions, which will be discussed below, they were also equally effective in raising ATP concentration. Acetyl-CoA (0.5 mM),

palmitoyl-CoA (10–50 μM), citrate (0.5–2 mM) and aspartate (5 mM) with oxoglutarate (5 mM) plus malate (0.5 mM) had no effect on the proportion of active dehydrogenase (results not shown). These studies failed to provide evidence for the specific effects of particular substrates in facilitating or interfering with the phosphorylation and inactivation of pyruvate dehydrogenase. In general the proportion of active dehydrogenase was inversely correlated with the mitochondrial concentration of ATP, but some important exceptions were noted, to which attention is drawn.

As shown in Table 6, succinate at concentrations

between 0.25 and 5 mM generated the same ATP concentration, whereas the proportion of active dehydrogenase was significantly greater at 0.25 than at 5 mM-succinate. The proportion of active dehydrogenase fell as palmitoyl-L-carnitine concentration was increased from 10 to 50 μM, whereas the concentration of ATP was little changed. n-Octanoate was as effective as 2-oxoglutarate plus malate in lowering pyruvate dehydrogenase activity but much less effective in raising the ATP concentration. Nevertheless the relatively small effect of octanoate on the ATP concentration was important to its effect on pyruvate dehydrogenase; in the presence of oligomycin, octanoate had little effect on either the ATP concentration or on pyruvate dehydrogenase activity. Oligomycin had little effect on ATP concentration or on pyruvate dehydrogenase with oxoglutarate plus malate, presumably because substrate-level phosphorylation at the level of succinate thiokinase was sufficiently rapid to maintain the ATP concentration. Fig. 2 shows the time-course of ATP concentration and of pyruvate dehydrogenase activity during the incubation of rat heart mitochondria. During incubation without substrate, pyruvate dehydrogenase activity rose during the second minute, whereas the ATP concentration did not fall until after the third minute. The time-course of inactivation of pyruvate dehydrogenase with

octanoate or succinate or 2-oxoglutarate plus malate as substrates was very similar; the activity fell during the first minute and remained constant thereafter. The ATP concentrations were different. With octanoate as substrate, the ATP concentration rose only slightly during the second minute and was not fully maintained. With oxoglutarate plus malate, or succinate, the ATP concentration rose substantially during the first minute; with oxoglutarate plus malate, the ATP concentration was maintained over 6 min; with succinate, the ATP concentration fell substantially between 2 and 6 min.

Discussion of the possible mechanism of these interesting anomalies is deferred to a later section.

Effects of NADH, NAD+, acetyl-CoA and CoA on the pyruvate dehydrogenase kinase reaction

Pyruvate dehydrogenase kinase activity was assayed as described by Cooper et al. (1974), except that in all of the present experiments the various potential effectors studied were added immediately before the initiation of the reaction with ATP. This choice of procedure was occasioned by the need to avoid possible oxidation of NADH by NADH oxidase activity in purified pyruvate dehydrogenase preparations. Control experiments showed that there was no detectable decrease in E_{340} of NADH during the period of the kinase assay. Unless otherwise

Table 7. *Effects of NAD+ and of NADH on the activity of pyruvate dehydrogenase kinase*

Pig heart pyruvate dehydrogenase (2 units/ml) was incubated at 30°C in 20 mM-potassium phosphate, 5 mM-2-mercaptoethanol, pH 7.0, with [γ-^{32}P]ATP (100 μM; 5–20 μCi/μmol) and 2 mM-MgCl$_2$. Other additions shown in the Table were made immediately before initiation of the reaction with ATP. The reaction was terminated and ^{32}P in protein-bound phosphate assayed as described by Cooper et al. (1974). The results are means ± S.E.M. for the numbers of assays in parentheses. For this preparation maximum incorporation was 1030 pmol/unit of dehydrogenase.

Expt.	Concentration (mM)		Kinase activity (pmol of ^{32}P incorporated/unit of dehydrogenase)		
	NAD+	NADH			
	Incubation time (min)		... 1.5	3	6
1	0	0	275 ± 5 (9)	467 ± 11 (6)	714 ± 23 (6)
	0	1	434 ± 12 (9)**	605 ± 9 (6)**	845 ± 5 (3)**
2	0	0	280 ± 1.3 (15)	—	—
	0	0.2	390 ± 2.1 (15)**	—	—
3	0	0	295 ± 9 (3)	518 ± 12 (3)	808 ± 25 (3)
	1	0	339 ± 23 (3)	531 ± 19 (3)	776 ± 25 (3)
	0	1	432 ± 2 (3)**	719 ± 19 (3)**	1064 ± 32 (3)
	1	1	463 ± 11 (3)**	736 ± 11 (3)**	1028 ± 32 (3)
4	0	0	193 ± 3.4 (9)	—	—
	0.05	0	192 ± 14 (3)	—	—
	0.10	0	193 ± 15 (3)	—	—
	0.25	0	198 ± 9 (3)	—	—
	1.0	0	195 ± 1 (3)	—	—
	0	0.005	259 ± 9 (3)**	—	—
	0	0.05	259 ± 11 (3)**	—	—
	0	0.1	257 ± 12 (3)**	—	—
	0	1.0	248 ± 7 (3)**	—	—

** $P < 0.01$ against zero NAD+, zero NADH; for other differences $P > 0.05$.

Table 8. *Effect of NAD⁺ on activation of the pyruvate dehydrogenase kinase reaction by NADH*

For conditions of assay see the legend to Table 7; the duration of assay was 1.5 min. Results are means ± s.e.m.; there were nine observations for zero NAD⁺, zero NADH, and three observations in all other groups.

Kinase activity (pmol of ^{32}P incorporated/unit of dehydrogenase)

[NAD⁺] (μM) ...	0	500	2000
[NADH] (μM)	219 ± 4	221 ± 2	228 ± 8
10	279 ± 7*	244 ± 3*‡	234 ± 4†
50	279 ± 6*	261 ± 15	242 ± 2†
100	269 ± 8*	252 ± 4*	258 ± 4
250	295 ± 3*	276 ± 7*	262 ± 3**†
500	278 ± 6*	278 ± 3*	261 ± 8
1000	270 ± 8*	277 ± 4*	257 ± 7

* $P < 0.01$ against zero NADH.
** $P < 0.05$ against zero NADH.
† $P < 0.01$ against zero NAD⁺.
‡ $P < 0.05$ against zero NAD⁺.
Other differences $P > 0.05$.

stated the preparations of pig heart pyruvate dehydrogenase used in these studies contained some thiamin pyrophosphate (sufficient to give approx. 15% of V_{max}. value in the absence of added thiamin pyrophosphate) and a small amount of residual phosphatase activity (sufficient to hydrolyse no more than 4% of the pyruvate dehydrogenase phosphate formed during the kinase assay).

As Table 7 shows, NADH alone activates the kinase reaction at concentrations ranging from 5 μM to 1 mM. The extent of activation (approx. 30–60%) was independent of the NADH concentration in this range, and was seen at all times of incubation from 1.5 to 6 min. NAD⁺ alone had no effects on the kinase reaction at concentrations ranging from 5 μM to 1 mM. NAD⁺ reverses this activation of the kinase reaction by NADH (Table 8). The results of these experiments and others not shown suggest that the effect of NAD⁺ is predominantly competitive, but the radioassay, being a stopped assay, is not sufficiently accurate for refined kinetic studies. As shown in Table 9, NADH also activates the kinase reaction in the presence of ADP (50–200 μM); ADP is a predominantly competitive inhibitor of the kinase reaction (competitive against ATP). NADP⁺ (0.2–1 mM) and NADPH (0.2–1 mM) had no effect on the kinase reaction (results not shown).

A number of initial attempts to demonstrate effects of acetyl-CoA on the pyruvate dehydrogenase kinase reaction, either alone or with NADH, showed considerable variation (Cooper *et al.*, 1974). In a typical series of five experiments with acetyl-CoA alone, activation of the kinase reaction was seen in two experiments, inhibition in two experiments and no

effect in the fifth; with NADH present, activation by acetyl-CoA was seen in one experiment, inhibition in one and no effect in the other two. Inhibition by CoA was, however, seen under both conditions. Subsequently evidence was obtained that enzyme-bound lipoate in pyruvate dehydrogenase is slowly reduced in the presence of 2-mercaptoethanol added to protect thiol groups in pyruvate dehydrogenase and its intrinsic kinase (Linn *et al.*, 1969a). This led to a re-investigation of effects of acetyl-CoA and of CoA in the presence of mixtures of NAD⁺ plus NADH. Entirely consistent effects of acetyl-CoA (and of CoA) have been seen in the presence of NAD⁺ plus NADH.

Table 10 shows effects of acetyl-CoA and of CoA (alone or together) on the pyruvate dehydrogenase kinase reaction with 2 mM-NAD⁺ plus either 10 μM- or 0.5 mM-NADH. As shown in the upper panel of Table 10, CoA inhibits the kinase reaction at 0.25 or 2 mM with either mixture of NAD⁺ plus NADH. As shown in the second panel of Table 10, acetyl-CoA (0.25 mM) activates the kinase reaction in the presence of either mixture of NAD⁺ plus NADH. Data given in the third panel of Table 10 shows that increasing concentrations of acetyl-CoA (0.25, 0.5 or 1 mM) produce graded re-activation of the kinase with CoA (2 mM) and 2 mM-NAD⁺ plus 0.5 mM-NADH. Finally, as shown in the lower panel of Table 10, the reversal of CoA inhibition by acetyl-CoA is dependent on the ratio [NADH]/[NAD⁺]. In other experiments (not shown) evidence was obtained that maximum activation of the kinase reaction could be achieved with NADH alone or with acetyl-CoA in the presence of non-activating or partially activating mixtures of NAD⁺ and NADH.

In summary, these results show that NADH activates the pyruvate dehydrogenase kinase reaction and that this effect of NADH can be reversed by

Table 9. *Effect of ADP on activation of the pyruvate dehydrogenase kinase reaction by NADH*

For conditions of assay see the legend to Table 7. The duration of assay was 1.5 min. Results are means ± s.e.m.; for zero ADP, zero NADH, there were nine observations, and for others three.

Kinase activity (pmol of ^{32}P incorporated/unit of dehydrogenase)

[ADP] (μM) ...	0	50	100	200
[NADH] (μM)				
0	133 ± 6	112 ± 3*	69 ± 1.4	56 ± 1.7
200	182 ± 7	147 ± 4	101 ± 2	80 ± 2

* $P < 0.05$ against zero ADP; for all other differences $P < 0.01$.

Table 10. *Interactions of CoA, acetyl-CoA, NAD^+ and NADH on the activity of pyruvate dehydrogenase kinase*

For conditions of assay see the legend to Table 7. The duration of assay was 1.5 min. Results are means ± S.E.M.; there were three observations in all groups except the first (zero concentrations), for which there were 12.

	Concentration (mM)				Kinase activity (pmol of ^{32}P incorporated/unit of dehydrogenase)
Expt.	NAD⁺	NADH	CoA	Acetyl-CoA	
1. Effect of CoA	0	0	0	0	127 ± 2
	2	0.01	0	0	132 ± 1
	2	0.01	0.25	0	98 ± 2*
	2	0.01	2.0	0	72 ± 1*
	2	0.5	0	0	137 ± 3
	2	0.5	0.25	0	117 ± 2*
	2	0.5	2.0	0	87 ± 1*
2. Effect of acetyl-CoA	2	0.01	0	0	132 ± 1
	2	0.01	0	0.25	152 ± 1†
	2	0.5	0	0	137 ± 3
	2	0.5	0	0.25	154 ± 1†
3. Effect of [acetyl-CoA]/ [CoA] ratio	2	0.5	2.0	0	87 ± 1
	2	0.5	2.0	0.25	130 ± 3‡
	2	0.5	2.0	0.50	143 ± 3‡
	2	0.5	2.0	1.00	154 ± 2‡
4. Effect of [NADH] on reversal of CoA inhibition by acetyl-CoA	2	0.01	2.0	0	72 ± 1
	2	0.01	2.0	0.25	69 ± 5
	2	0.5	2.0	0	87 ± 1
	2	0.5	2.0	0.25	97 ± 1§

* $P < 0.01$ against zero CoA.
† $P < 0.01$ against zero acetyl-CoA.
‡ $P < 0.01$ against zero acetyl-CoA.
§ $P < 0.01$ against zero acetyl-CoA.

NAD⁺. In the presence of mixtures of NAD⁺ plus NADH which produce no or partial activation of the kinase reaction, acetyl-CoA also activates the kinase reaction; CoA is inhibitory; and acetyl-CoA reverses the CoA inhibition. Reversal of CoA inhibition by acetyl-CoA is most marked at higher [NADH]/[NAD⁺] ratios. The kinase reaction, like the dehydrogenase reaction, is thus sensitive to [NADH]/[NAD⁺] and [acetyl-CoA]/[CoA] ratios.

Table 11 shows the effects of pyruvate on the kinase reaction in the absence and in the presence of NADH. In the absence of NADH low concentrations of pyruvate (50 or 100 μM) tended to activate the kinase reaction, whereas higher concentrations (250–1000 μM) were inhibitory; these findings are in keeping with those of Cooper *et al.* (1974) for preparations of pyruvate dehydrogenase contaminated with thiamin pyrophosphate. In the presence of NADH all concentrations of pyruvate inhibited the kinase reaction.

Concentrations of NADH, NAD⁺, acetyl-CoA and CoA in rat heart mitochondria

In the section concerned with effects of respiratory substrates some important exceptions were noted to a more general finding that the activity of pyruvate dehydrogenase (active form) is inversely correlated

Table 11. *Effect of NADH or pyruvate or of NADH plus pyruvate on the activity of pig heart pyruvate dehydrogenase kinase*

The conditions of assay and of incubation were as given in Table 7. The period of incubation was 1.5 min. Results are means ± S.E.M.; there were three observations in each group.

	Kinase activity (pmol of ^{32}P incorporated/unit of dehydrogenase)	
Pyruvate concn. (μM)	Control	0.2 mM-NADH
0	123 ± 5	176 ± 5†
50	142 ± 3	142 ± 1**
100	131 ± 2	132 ± 2**
250	113 ± 1	110 ± 2**
500	94 ± 1**	88 ± 1**†
750	85 ± 2**	78 ± 1**
1000	77 ± 1*	72 ± 1**

† $P < 0.01$ against zero NADH.
** $P < 0.01$ against zero pyruvate.
* $P < 0.001$ against zero pyruvate.

with the ATP concentration in isolated mitochondria. In view of the effects of NADH, NAD⁺, acetyl-CoA and CoA on the phosphorylation of pyruvate

Table 12. *Concentrations of NADH, NAD+, acetyl-CoA and CoA in rat heart mitochondria*

Rat heart mitochondria (1.5 mg) were incubated for 5 min at 30°C in 0.5 ml of KCl medium and reaction was terminated by the addition of 0.3 ml of 1 M-KOH in ethanol (for assay of NADH) or $10 \mu l$ of $HClO_4$ (assays of NAD+, CoA, acetyl-CoA). NADH and NAD+ were assayed against NADH and NAD+ standards with NADH luciferase. CoA and acetyl-CoA were assayed as NADH against CoA, acetyl-CoA and NADH standards with NADH luciferase (see the Experimental section for details). In this experiment acetyl-CoA was converted into CoA with L-carnitine plus carnitine acetyltransferase in place of oxaloacetate plus citrate synthase. Results are means ± s.e.m.; there were six assays in each group (two per mitochondrial incubation).

Mitochondrial incubation, additions	Concn. (nmol/mg of mitochondrial protein)				[NADH]/ [NAD+]	[Acetyl-CoA]/ [CoA]
	NADH	NAD+	Acetyl-CoA	CoA		
None	0.15 ± 0.02	6.60 ± 0.39	0.28 ± 0.09	0.89 ± 0.08	0.02	0.24
5 mM-2-oxoglutarate + 0.5 mM-L-malate	6.57 ± 0.38	3.20 ± 0.19	0.26 ± 0.12	0.70 ± 0.10	2.05	0.37
50μM-*n*-octanoate	1.35 ± 0.06	5.23 ± 0.82	0.75 ± 0.09	0.30 ± 0.08	0.26	2.50
10μM-palmitoylcarnitine + 0.5 mM-malate	0.26 ± 0.01	7.2 ± 0.30	—	—	0.04	—
20μM-palmitoylcarnitine + 0.5 mM-malate	0.64 ± 0.01	7.5 ± 0.14	0.40 ± 0.11	0.64 ± 0.10	0.09	0.63
50μM-palmitoylcarnitine + 0.5 mM-malate	1.19 ± 0.32	6.4 ± 0.10	0.47 ± 0.14	0.54 ± 0.18	0.19	0.87
0.25 mM-succinate	0.31 ± 0.004	7.2 ± 0.17	—	—	0.04	—
5 mM-succinate	3.4 ± 0.05	1.3 ± 0.15	—	—	2.62	—

dehydrogenase by its intrinsic kinase it seemed important to investigate the effects of respiratory substrates on the mitochondrial concentrations of these compounds. Methods have therefore been developed for the assay of NADH, NAD+, acetyl-CoA and CoA in extracts of rat heart mitochondria based on NADH luciferase. With good batches of enzyme such methods are highly sensitive and can assay NADH in the range 2–50 pmol. These results are shown in Table 12.

The concentration of (NAD+ plus NADH) in rat heart mitochondria averaged 7.3 nmol/mg of mitochondrial protein, which is somewhat greater than the value of 4.5 nmol/mg derived from the data of La Noue *et al.* (1973). The concentration of acid-soluble CoA plus acetyl-CoA was approx. 1.1 nmol/mg of protein. The ratio [NADH]/[NAD+] in mitochondria incubated without substrate was extremely low and was increased more than 100-fold by incubation with oxoglutarate plus malate. Octanoate, which was highly effective in promoting the inactivation of pyruvate dehydrogenase but which caused only a modest increase in the ATP concentration (Table 6), produced tenfold changes in the ratios [NADH]/[NAD+] and [acetyl-CoA]/[CoA]; it is suggested that these changes in ratio, through effects on the kinase reaction, may provide an explanation for the effects of octanoate on the activity of pyruvate dehydrogenase in rat heart mitochondria. Succinate at 5 mM was much more effective than at 0.25 mM in promoting inactivation of pyruvate dehydrogenase, although

both concentrations were equally effective in raising the ATP concentration (Table 6). As shown in Table 12, 0.25 mM-succinate produced very little change in the ratio [NADH]/[NAD+] as compared with the no-substrate control, whereas 5 mM-succinate increased the ratio by 130-fold. If the data with the pig heart pyruvate dehydrogenase kinase reaction are applicable to the rat heart enzyme, such a change in ratio in the vicinity of the pyruvate dehydrogenase kinase reaction could account for the greater effectiveness of 5 mM-succinate in promoting the inactivation of the enzyme. The data in Table 6 show that increasing concentrations of palmitoylcarnitine (from 10 to 20 to 50μM) led to increased inactivation of pyruvate dehydrogenase with only small changes in the ATP concentration. Table 12 shows increasing [NADH]/[NAD+] ratios and some increase in [acetyl-CoA]/[CoA] as palmitoylcarnitine was increased in concentration.

These data with octanoate, succinate and palmitoylcarnitine thus provide some evidence that ratios of [NADH]/[NAD+] and [acetyl-CoA]/[CoA] may regulate the pyruvate dehydrogenase kinase reaction in rat heart mitochondria. It will be important to show that these ratios modulate the kinase reaction in the rat heart pyruvate dehydrogenase complex, but current methods of purification of the complex are not adequate for this purpose. It will also be necessary to develop sensitive methods for measuring the ADP concentration in mitochondria and for varying independently the ratios [ATP]/[ADP], [NADH]/

[NAD$^+$] and [acetyl-CoA]/[CoA] in order to define more closely the role of these ratios in the regulation of the phosphorylation and dephosphorylation of pyruvate dehydrogenase at the level of the intact mitochondrion.

Previous studies of the effects of respiratory substrates, external ADP, uncouplers of oxidative phosphorylation and inhibitors of adenine nucleotide translocation on the activity of pyruvate dehydrogenase in isolated fat-cell and liver and heart mitochondria have been interpreted solely in terms of their effects on the concentration of ATP or on ratios of [ATP]/[ADP] (Martin et al., 1972; Cooper et al., 1974; Whitehouse et al., 1974; Wieland & Portenhauser, 1974; Chiang & Sacktor, 1975). It seems possible that these interpretations may be incomplete if ratios of [NADH]/[NAD$^+$] and [acetyl-CoA]/[CoA] also affect the pyruvate dehydrogenase kinase reaction in fat-cell and liver mitochondria. Taylor et al. (1975), in an extensive study of the relation between [ATP]/[ADP] ratios and pyruvate dehydrogenase activity in rat liver mitochondria, observed that NH$_4$Cl, glutamate, 3-hydroxybutyrate, acetoacetate or octanoate could change the mitochondrial pyruvate dehydrogenase activity without altering the ratio of [ATP]/[ADP]. They concluded that the mitochondrial [NADH]/[NAD$^+$] ratio might be important and that high ratios of [NADH]/[NAD$^+$] might facilitate phosphorylation and inactivation of pyruvate dehydrogenase. Häussinger et al. (1975) observed that metabolism of NH$_4^+$ in rat liver could increase the activity of pyruvate dehydrogenase and suggested that this could be due to a decreased ratio of [NADH]/[NAD$^+$] as a result of glutamate synthesis via glutamate dehydrogenase. If our findings are applicable to the rat liver enzyme then they provide some support for these interpretations. However, these interpretations may also be incomplete because they overlook the possible contribution of the ratio [acetyl-CoA]/[CoA].

The phosphorylation and inactivation of pyruvate dehydrogenase is favoured by high ratios of [ATP]/[ADP], of [NADH]/[NAD$^+$] and of [acetyl-CoA]/[CoA] (Linn et al., 1969a,b; Wieland & Portenhauser, 1974; Cooper et al., 1974; the present study). The reversibility of the first site of oxidative phosphorylation (Chance & Hollunger, 1961; Ernster & Lee, 1964) and evidence for equilibration between adenine nucleotides and the cytochrome system (Klingenberg & Schollmeyer, 1961; Slater, 1971; Wilson et al., 1972a,b) indicate that the [NADH]/[NAD$^+$] and [ATP]/[ADP] ratios may be directly related. There is thus the basis for an amplification step in regulation of pyruvate dehydrogenase phosphorylation by these two concentration ratios. The lack of a sufficiently sensitive assay for mitochondrial ADP thus far prevents us from giving complete evidence on this point.

General Discussion and Conclusions

Effects of metabolism of fatty acids and ketone bodies and of diabetes on pyruvate oxidation and on pyruvate dehydrogenase activity in rat heart

The observations that high ratios of [acetyl-CoA]/[CoA] and of [NADH]/[NAD$^+$] are inhibitors of pyruvate dehydrogenase and that the metabolism of fatty acids or of ketone bodies, or the development of alloxan-diabetes, elevates the ratio of [acetyl-CoA]/[CoA] in perfused rat heart led to the suggestion that effects of this ratio on pyruvate dehydrogenase may mediate inhibitory effects of these agents on oxidation of pyruvate formed from glucose in this tissue (Garland & Randle, 1964a,b; Randle et al., 1966). The rapidity and extent of the change in [acetyl-CoA]/[CoA] ratio and its correlation with rapid onset of inhibition of pyruvate oxidation in perfusions with acetate supported this conclusion (Randle et al., 1970). The discovery by Wieland et al. (1971a,b) that perfusion with fatty acids or with ketone bodies or the development of alloxan-diabetes is associated with phosphorylation and inactivation of pyruvate dehydrogenase suggested the possibility that the ratio [acetyl-CoA]/[CoA] might also regulate the phosphorylation and inactivation of pyruvate dehydrogenase. This expectation has been realized in the present study, where it has been shown that increasing the ratio of [acetyl-CoA]/[CoA] activates pyruvate dehydrogenase kinase at a suitable ratio of [NADH]/[NAD$^+$]. It seems a reasonable working hypothesis to suggest that fatty acid and ketone-body metabolism may inactivate and inhibit pyruvate dehydrogenase by elevating the mitochondrial ratio of [acetyl-CoA]/[CoA] in the perfused heart. However, information about mitochondrial ratios of [acetyl-CoA]/[CoA] and of [NADH]/[NAD$^+$] in the perfused heart are necessary for proof of this hypothesis. Palmitoyl-L-carnitine failed to promote phosphorylation and inactivation of pyruvate dehydrogenase in rat heart mitochondria under conditions where pyruvate oxidation was inhibited and the ratio of [acetyl-CoA]/[CoA] was markedly increased. The reason for this is not known, but it suggests a need for more refined kinetic studies of the relationship between end-product inhibition of the dehydrogenase and its phosphorylation through the kinase reaction. In the perfused heart, acetate oxidation totally suppressed oxidation of pyruvate formed from glucose (Randle et al., 1970), whereas acetate oxidation does not result in complete inactivation of pyruvate dehydrogenase by phosphorylation (Whitehouse et al., 1974; the present study). This could imply that end-product inhibition can occur without complete phosphorylation of the dehydrogenase.

The present study has suggested that the effects of diabetes on pyruvate dehydrogenase are more complex than those of an acute increase in fatty acid

oxidation. Thus diabetes induces changes that impair inhibitory effects of pyruvate and of dichloroacetate on the phosphorylation of pyruvate dehydrogenase by its intrinsic kinase. Acute effects of fatty acids may be much less effective in this respect than those of diabetes. An additional mechanism or mechanisms may thus be involved in the diabetic tissue. An impaired ability of mitochondria to accumulate dichloroacetate or pyruvate in diabetes could be one factor involved.

Mechanism of effects of NADH, NAD$^+$, acetyl-CoA and CoA on the pyruvate dehydrogenase kinase reaction

NADH, NAD$^+$, acetyl-CoA and CoA are all substrates or products of the pyruvate dehydrogenase reactions shown in the introduction to this paper.

The substrate sites for NADH and for NAD$^+$ are located on Enz$_3$ (dihydrolipoyl dehydrogenase), whereas those for acetyl-CoA and CoA are located on Enz$_2$ (dihydrolipoate acetyltransferase). Studies in Dr. L. J. Reed's laboratory by Linn *et al.* (1972) have indicated that the pyruvate dehydrogenase complex consists of a core of acetyltransferase to which are attached the dihydrolipoyl dehydrogenase, the kinase and the substrate for the kinase (Enz$_1$, pyruvate decarboxylase). They have also shown that the activity of the kinase is markedly enhanced by its attachment to the acetyltransferase and that it phosphorylates a serine residue in the α-subunit of the decarboxylase (Enz$_1$). These considerations raise a number of questions about the mechanism of effects of NADH, NAD$^+$, acetyl-CoA and CoA. If these compounds affect the kinase reaction by binding to their substrate sites on the acetyltransferase or on the dihydrolipoyl dehydrogenase then these effects must be transmitted to subunits of the kinase or of its substrate, the decarboxylase. The mechanism might involve conformational changes, in view of the importance of attachment to the acetyltransferase for activity of the kinase. Another possibility would be the presence of regulator sites for these four effectors on the kinase or its substrate. A third possibility of novel interest is that lipoate may transmit these regulatory interactions. Reed (1969) has pointed out that lipoate visits each of the three enzymes involved in the course of conversion of pyruvate, CoA and NAD$^+$ into acetyl-CoA, NADH and CO$_2$ by the complex. Thus there are likely to be binding sites for lipoate in its various forms on each of the three enzymes. It is possible that the position in which lipoate is parked depends on its state (i.e. whether oxidized, reduced or acetylated) and that its position may influence phosphorylation of the decarboxylase. Thus as a matter of speculation, the location of lipoate on the decarboxylase might inhibit phosphorylation through steric hindrance or by some other mechanism. With NADH, reduced lipoate is formed

and may be located on Enz$_3$, facilitating phosphorylation. With NAD$^+$, oxidized lipoate may be formed by switching location to Enz$_1$. With acetyl-CoA and NADH, acetylhydrolipoate may be formed by switching location to Enz$_2$. Similarly, low concentrations of pyruvate plus thiamin pyrophosphate, or of acetoin plus thiamin pyrophosphate, may facilitate phosphorylation (Cooper *et al.*, 1974), by causing the location of lipoate to switch to Enz$_2$. The mechanism of action of these effectors could be of particular interest in mechanisms of enzyme regulation in multi-enzyme complexes in general and merits further study.

Regulation of enzymes by metabolites and by phosphorylation and dephosphorylation

The phosphorylation and inactivation of pyruvate dehydrogenase by its intrinsic kinase is facilitated by metabolites which are also inhibitors of the dehydrogenase reaction (acetoin, acetyl-CoA, NADH) and is inhibited by substrates for the full dehydrogenase reaction (pyruvate, thiamin pyrophosphate, CoA and NAD$^+$). Thus metabolites that inhibit the dehydrogenase facilitate its inactivation, and vice versa. This type of dual regulation is well recognized in the case of glycogen phosphorylase (EC 2.4.1.1) and of UDP-glucose–glycogen glucosyltransferase (EC 2.4.1.11), which differ from pyruvate dehydrogenase in that the *b* form can also be activated by allosteric effectors. Thus, for example, muscle glycogen phosphorylase *b* is activated allosterically by 5'-AMP, which may also facilitate the conversion of phosphorylase *b* into phosphorylase *a* by phosphorylation through inhibition of phosphorylase *a* phosphatase. With UDP-glucose–glycogen glucosyltransferase, glucose 6-phosphate may be an activator of the *b* and the *a* forms and may also facilitate the conversion of the *b* form into the *a* form through dephosphorylation (review by Soderling & Park, 1974). There is evidence that these effects of 5'-AMP (phosphorylase) and glucose 6-phosphate (transferase) on phosphorylation and dephosphorylation are due to effects on the protein substrate (i.e. phosphorylase or transferase) and not to effects on the kinase or the phosphatase. It is possible that the effects of metabolites on the pyruvate dehydrogenase and pyruvate dehydrogenase kinase reactions may be exerted through common binding sites, though this is a point on which there is little evidence at the present time.

Thus in the case of three key enzymes of carbohydrate metabolism there is evidence that metabolite effectors of the enzyme may also modulate covalent modification by phosphorylation or dephosphorylation; inhibitory metabolites facilitate inactivation and vice versa. Ljungström *et al.* (1974) and Hjelmquist *et al.* (1974) have described the phosphorylation of liver pyruvate kinase by cyclic AMP-dependent

protein kinase, and Carlsson & Kim (1974) have described the phosphorylated and dephosphorylated forms of acetyl-CoA carboxylase. It seems possible that the regulation of mammalian enzymes by phosphorylation and dephosphorylation may be more common than has been considered and that the dual modality of metabolite regulation which is exemplified by phosphorylase, UDP-glucose-glycogen glucosyltransferase and now pyruvate dehydrogenase may find further extensions.

Since this paper was submitted we have received the paper by Pettit *et al.* (1975) describing activation of ox heart pyruvate dehydrogenase kinase reaction by acetyl-CoA and by NADH and inhibition by CoA and NAD^+. Our findings agree with their conclusion that increasing ratios of $[NADH]/[NAD^+]$ and of $[acetyl-CoA]/[CoA]$ favour phosphorylation and inactivation of pyruvate dehydrogenase. There are, however, some differences in detail in that we have been unable to show convincing activation of the kinase reaction by acetyl-CoA in the absence of mixtures of NAD^+ plus NADH, or inhibition by NAD^+ in the absence of NADH, or effects of acetyl-CoA at saturating concentrations of NADH.

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