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# Diabetes and the Control of Pyruvate Dehydrogenase in Rat Heart Mitochondria by Concentration Ratios of Adenosine Triphosphate/Adenosine Diphosphate, of Reduced/Oxidized Nicotinamide–Adenine Dinucleotide and of Acetyl-Coenzyme A/Coenzyme A

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1. The proportion of active (dephosphorylated) pyruvate dehydrogenase in rat heart mitochondria was correlated with total concentration ratios of ATP/ADP, NADH/$NAD^+$ and acetyl-CoA/CoA. These metabolites were measured with ATP-dependent and NADH-dependent luciferases. 2. Increase in the concentration ratio of NADH/$NAD^+$ at constant [ATP]/[ADP] and [acetyl-CoA]/[CoA] was associated with increased phosphorylation and inactivation of pyruvate dehydrogenase. This was based on comparison between mitochondria incubated with 0.4mM- or 1mM-succinate and mitochondria incubated with 0.4mM-succinate±rotenone. 3. Increase in the concentration ratio acetyl-CoA/CoA at constant [ATP]/[ADP] and [NADH]/[$NAD^+$] was associated with increased phosphorylation and inactivation of pyruvate dehydrogenase. This was based on comparison between incubations in 50$\mu$M-palmitoyl-L-carnitine and in 250$\mu$M-2-oxoglutarate+50$\mu$M-L-malate. 4. These findings are consistent with activation of the pyruvate dehydrogenase kinase reaction by high ratios of [NADH]/[$NAD^+$] and of [acetyl-CoA]/[CoA]. 5. Comparison between mitochondria from hearts of diabetic and non-diabetic rats shows that phosphorylation and inactivation of pyruvate dehydrogenase is enhanced in alloxan-diabetes by some factor other than concentration ratios of ATP/ADP, NADH/$NAD^+$ or acetyl-CoA/CoA.

Pyruvate dehydrogenase (EC 1.2.4.1) of animal tissues is inhibited by its end products acetyl-CoA (competitive with CoA), NADH (competitive with $NAD^+$) and acetoin (competitive with pyruvate) (Garland & Randle, 1964*a*; Cooper *et al.*, 1974). The enzyme complex is also regulated by interconversion of dephosphorylated (active) and phosphorylated (inactive) forms catalysed by pyruvate kinase and pyruvate dehydrogenase phosphate dehydrogenase phosphatase (Linn *et al.*, 1969*a*,*b*). In rat heart, increased conversion of the enzyme into the phosphorylated (inactive) form occurs in alloxan-diabetes or on perfusion with fatty acids and ketone bodies. Formation of the dephosphorylated (active) form is increased by perfusion with pyruvate. This effect of pyruvate is substantially inhibited in perfused hearts from alloxan-diabetic rats (Kerbey *et al.*, 1976). The effect of alloxan-diabetes in facilitating the phosphorylation and inactivation of pyruvate dehydrogenase persists in mitochondria prepared from hearts of diabetic animals; the proportion of inactive enzyme is increased on incubation with (2-oxoglutarate+malate) or (2-oxoglutarate+malate+pyruvate) (Kerbey *et al.*, 1976).

Some explanation for these findings in the perfused heart is provided by studies of the kinase reaction in the purified pyruvate dehydrogenase complex from bovine kidney and pig heart. These studies showed that phosphorylation and inactivation of the complex is facilitated by high ratios of [ATP]/[ADP], [NADH]/[$NAD^+$] and [acetyl-CoA]/[CoA] and inhibited by pyruvate (Linn *et al.*, 1969*b*; Pettit *et al.*, 1975; Cooper *et al.*, 1975; Kerbey *et al.*, 1976). The concentration ratio of acetyl-CoA/CoA is increased in rat heart by diabetes or by oxidation of fatty acids and ketone bodies. However, negative findings with respect to regulation of the kinase reaction by the concentration ratios of NADH/$NAD^+$ and of acetyl-CoA/CoA have been reported (Siess & Wieland, 1976). Further, it has been suggested that some additional mechanism may be involved in the effects of diabetes (Kerbey *et al.*, 1976).

In an earlier paper (Kerbey *et al.*, 1976), some preliminary data were given which suggested that the concentration of active (dephosphorylated) pyruvate dehydrogenase in rat heart mitochondria was influenced by the concentration ratios of NADH/$NAD^+$ and of acetyl-CoA/CoA. In the present study the effect of isolated variation in the ratio of either [NADH]/[$NAD^+$] or [acetyl-CoA]/[CoA] on the proportion of active (dephosphorylated) pyruvate dehydrogenase in rat heart mitochondria has been investigated. The ratios of [ATP]/[ADP], [NADH]/[$NAD^+$] and [acetyl-CoA]/[CoA] have been compared in heart mitochondria from non-diabetic and diabetic rats and correlated with the proportion of active pyruvate dehydrogenase.

In order to study the effect of variation in ratios of [NADH]/[NAD$^+$] and of [acetyl-CoA]/[CoA] we have used the empirical approach of trying a number of combinations of respiratory substrates and inhibitors. An alternative approach was to use extramitochondrial 3-hydroxybutyrate and acetoacetate to buffer intramitochondrial [NADH]/[NAD$^+$] through 3-hydroxybutyrate dehydrogenase, and to use extramitochondrial acetyl-L-carnitine and L-carnitine to buffer intramitochondrial [acetyl-CoA]/[CoA] through carnitine acetyltransferase. This proved impracticable, as the activity of 3-hydroxybutyrate dehydrogenase was too low to permit buffering of [NADH]/[NAD$^+$] in respiring rat heart mitochondria. Acetyl-L-carnitine at concentrations that substantially increased the mitochondrial ratio of [acetyl-CoA]/[CoA] lowered the ratio of [ATP]/[ADP]. We have therefore used the empirical approach. To ensure that only a small fraction of substrates and oxygen was used during incubation, concentrations of mitochondria were kept below 2 mg of mitochondrial protein/ml. This made it necessary to use very sensitive methods for assay of metabolites based on ATP-dependent and NADH-dependent luciferases and suitable adaptations and improvements to these are described. In these experiments it is the total mitochondrial concentrations of ATP, ADP, NADH, NAD$^+$, acetyl-CoA and CoA that were measured. In what follows it is assumed that the total concentrations of these metabolites may reflect their free concentrations.

Batenburg & Olson (1975, 1976) have published evidence that an increased ratio of [NADH]/[NAD$^+$] or of [acetyl-CoA]/[CoA] can facilitate inactivation of pyruvate dehydrogenase in rat liver mitochondria. However, in these experiments high concentrations of mitochondria were used (3.5–5 mg of mitochondrial protein/ml) and since ADP, glucose and hexokinase were present high rates of substrate utilization were presumably maintained. Measurements of metabolite concentrations and of pyruvate dehydrogenase activity were only made at a single time of incubation (10 min). The total concentration of pyruvate dehydrogenase (sum of inactive and active forms) was not measured. Moreover, inactivation of pyruvate dehydrogenase by proteolysis is pronounced in extracts of rat liver mitochondria unless precautions such as inclusion of rat serum or removal of lysosomes with digitonin are observed (Wieland, 1975). We have attempted to overcome these objections in the present study by using lower concentrations of mitochondria, by making measurements of metabolites and of pyruvate dehydrogenase at least twice during incubation and by including incubations in which total pyruvate dehydrogenase was assayed. Inactivation of pyruvate dehydrogenase by proteolysis in extracts of rat heart mitochondria is not a problem under the conditions that we have used, as enzyme activity is stable for several hours at 0°C and for at least 10 min at 30°C.

Hansford (1976) has reported that the proportion of active pyruvate dehydrogenase in rat heart mitochondria is decreased by increasing ratios of [ATP]/[ADP] or [NADH]/[NAD$^+$] or [acetyl-CoA]/[CoA]. In these experiments ratios were imposed by use of external ATP+ADP with uncouplers plus oligomycin; with acetoacetate+3-hydroxybutyrate with rotenone; and with acetyl-DL-carnitine+DL-carnitine. We have avoided this particular approach for a number of reasons. First, we wished to avoid the use of a number of inhibitors. Secondly, we did not wish to use acetyl-L-carnitine because it has unexplained effects on ATP concentration. Thirdly, use of external ATP+ADP may involve assumptions regarding the activity of the adenine nucleotide translocase which may be inhibited by long-chain acyl-CoA. For these reasons we have chosen the tedious but more physiological approach of using various combinations of respiratory substrates.

## Experimental

### *Materials*

Biochemical reagents and enzymes were purchased from Boehringer Corp. (London) Ltd., London, W.5, U.K., or from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K., with the following exceptions. Alloxan and L-carnitine were from Koch–Light Laboratories, Colnbrook, Bucks., U.K. Palmitoyl-L-carnitine was synthesized by the method of Bremer (1962). It was shown to be substantially free of L-carnitine (criteria of its purity are given in Kerbey *et al.*, 1976). Acetyl-CoA was synthesized by the method of Simon & Shemin (1953). Tetradecyl aldehyde was from Ralph N. Emmanuel Ltd., Wembley, Middx., U.K.

Pyruvate dehydrogenase phosphate phosphatase was prepared as described by Denton *et al.* (1972). 2-Oxoglutarate dehydrogenase (EC 1.2.4.2) was purified from pig heart as described by Kerbey *et al.* (1976). Arylamine acetyltransferase (EC 2.3.1.5) was purified from pigeon liver acetone-dried powder by the method of Tabor *et al.* (1953).

### *Methods*

*Rats.* Hearts were obtained from male albino Wistar rats (300–400*g*) which were allowed free access to water and to diet 41B (Oxoid, London S.E.1, U.K.). Diabetes was induced by the intravenous injection of alloxan (60 mg/kg) into a tail vein under diethyl ether anaesthesia and was confirmed 40–48 h later by monitoring the concentration of blood glucose with Dextrostix (Miles Laboratories, Slough, Bucks., U.K.) at the time of removal of the heart.

*Heart mitochondria: preparation and incubation.* Heart mitochondria were prepared with use of the Polytron PT10 homogenizer as described by Kerbey *et al.* (1976), and quantified by assay of mitochondrial protein (Gornall *et al.*, 1949). Mitochondria were incubated at 30°C in Eppendorf tubes in KCl medium (120mM-KCl, 20mM-Tris/HCl, 5mM-potassium phosphate, 2mM-EGTA, pH7.4) at a concentration of 1mg of mitochondrial protein/0.5ml. Details of other additions and times of incubation are given in the text, Tables or Figures. For assay of adenine nucleotides, acetyl-CoA, CoA and $NAD^+$, incubations were terminated by addition of 70% (v/v) $HClO_4$ (10$\mu$l/ml). For assay of NADH, incubations were terminated by addition of 1M-KOH in ethanol (0.6ml/ml). For assay of pyruvate dehydrogenase, mitochondria were separated by centrifuging for 20s in an Eppendorf 3200 centrifuge, the supernatant was aspirated and the pellet frozen in liquid nitrogen. The extracts were prepared for assay of these substances as described by Kerbey *et al.* (1976).

*Assay of pyruvate dehydrogenase.* Pyruvate dehydrogenase (active, dephosphorylated form) was assayed spectrophotometrically in extracts of heart mitochondria by coupling to arylamine acetyltransferase as described by Coore *et al.* (1971). For assay of total pyruvate dehydrogenase (sum of active plus inactive forms) the mitochondrial pellets were dispersed by ultrasonic disintegration into 250$\mu$l of 50mM-Tris / HCl / 2mM-EDTA / 1mM-dithiothreitol, pH7.0. Conversion of inactive (phosphorylated) pyruvate dehydrogenase into the active form was effected by incubation for 10min at 30°C of 20$\mu$l of mitochondrial extract with 10$\mu$l of 10mM-$CaCl_2$/250mM-$MgSO_4$ and 10$\mu$l of pyruvate dehydrogenase phosphate phosphatase (sufficient to complete conversion within 10min).

*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 incubation medium with $HClO_4$. Control experiments showed that standard curves were linear over the range 10–200pmol.

ADP was measured after conversion into ATP by a modification of the method of Kimmick *et al.* (1975). Portions (10$\mu$l) of $HClO_4$ extracts of mitochondria were incubated in ATP-assay buffer (Stanley & Williams, 1969) at room temperature containing 100mM-KCl, 0.25mM-phosphoenolpyruvate and 1.5 units of pyruvate kinase. (All enzyme activities are given as EC units, i.e. 1$\mu$mol of substrate converted/min.) Control experiments showed that conversion of ADP into ATP is complete within 1min and that the ATP so formed is stable at room temperature for at least 45min before assay; assays were completed well within this time. Control experiments with standard ADP (1 and 4nmol) added to $HClO_4$ extracts of mitochondria showed the recovery of ADP to be approx. 80%. The values given were not corrected for recovery. NADH, $NAD^+$ (after conversion into NADH), CoA and acetyl-CoA (assayed as NADH formed in the 2-oxoglutarate dehydrogenase reaction) were assayed with NADH-dependent luciferase (Stanley, 1971) essentially as described by Kerbey *et al.* (1976). The sensitivity and reproducibility of these assays was improved in the present study by modifications described below.

NADH (10–20pmol) was assayed in 5ml disposable plastic tubes which contained 1ml of 100mM-potassium phosphate buffer, pH7, and 5mM-2-mercaptoethanol. The following additions were made sequentially: 1ml of 100mM-potassium phosphate buffer, pH7, containing 5mM-2-mercaptoethanol and 10$\mu$l of FMN (4mg/ml in water) was added from a dark bottle with a dispensing attachment (storage of FMN in the dark before the assay improved the sensitivity and reproducibility of the assays such as to detect 2pmol of NADH). Then 20$\mu$l of tetradecyl aldehyde (0.1%, w/v, in methanol) was added, followed by 5–10$\mu$l of NADH-dependent luciferase (10mg/ml in water). The contents of the tube were then mixed and the light emitted by the luciferase system was measured in a Nuclear–Chicago Isocap 300 liquid-scintillation spectrometer 1min after addition of the enzyme. Each sample was counted for 0.2min. Control experiments with standard NADH solutions showed the assay to be linear over the range 0–100pmol. NADH-dependent luciferase will also respond to NADPH. For the determination of d.p.m. corresponding to NADPH, and hence of NADH by difference, each sample was re-assayed 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 conversion of NADH into $NAD^+$ was complete within 1min. Control experiments with standard NADH (1 and 2nmol) added to ethanolic KOH extracts of mitochondria showed the recovery of NADH to be approx. 98%. NADH standards (3nmol) were incorporated in parallel with each series of incubations and the concentration of NADH in mitochondrial extracts was computed with respect to the recovery of standard NADH. NADH solutions used in these experiments were standardized spectrophotometrically at 340nm.

$NAD^+$ was assayed after conversion into NADH with ethanol and ethanol dehydrogenase. The procedure was as follows: 100$\mu$l of $HClO_4$ extracts of mitochondria (or 3nmol standard of $NAD^+$ in $HClO_4$-treated KCl medium) were added at room temperature to 0.9ml of 75mM-semicarbazide/200mM-glycine/150mM-ethanol, pH10, and 2.5 units of yeast ethanol dehydrogenase (EC 1.1.1.1) was added. Control experiments showed that the con-

version of $NAD^+$ into NADH was complete within 1–2min. The NADH formed was assayed in a sample containing 20–40pmol of NADH. Control experiments showed that NADH in ethanolic KOH and $NAD^+$ in $HClO_4$ were stable for at least 4h at 4°C before assay; assays were completed well within this time.

CoA was assayed as NADH formed in the 2-oxoglutarate dehydrogenase reaction. Acetyl-CoA was assayed as CoA released from acetyl-CoA by carnitine acetyltransferase and carnitine [carnitine acetyltransferase can also release CoA from short-chain acyl-CoA; however, these do not accumulate in mitochondria in significant amount during β-oxidation (Stanley & Tubbs, 1975)]. The procedure was as follows: 0.6ml of $HClO_4$ extracts of mitochondria was added to 1.8ml of 100mM-potassium phosphate buffer, pH7, containing 5mM-2-mercaptoethanol, 2mM-$MgCl_2$ and 75μl of 1M-KOH. The final pH was 6.9–7.1. For the assay of CoA, 0.5ml of this mixture of $HClO_4$ extract and buffer was incubated at 30°C for 6min with $NAD^+$ (to 0.21mM), thiamin pyrophosphate (to 0.78mM), 2-oxoglutarate (to 2.5mM) and 40–50munits of 2-oxoglutarate dehydrogenase. The reaction was complete within 5min 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 L-carnitine (to 5mM) and 20munits of carnitine acetyltransferase. NADH produced was assayed by the NADH-dependent luciferase assay in a sample containing 20–40pmol of NADH (see above); control experiments showed that the NADH formed in the 2-oxoglutarate dehydrogenase reaction was stable for at least 45min; assays were completed well within this time. CoA (3nmol) and acetyl-CoA (3nmol) standards in $HClO_4$-treated KCl medium were incorporated in parallel with the experimental samples and corrections for their recoveries were applied when computing the mitochondrial concentrations of CoA and acetyl-CoA. Control experiments with CoA (0.7nmol) and acetyl-CoA (0.7nmol) in $HClO_4$ extracts of mitochondria showed their recoveries to be 98 and 85% respectively. For each experiment, stock solutions of CoA and acetyl-CoA were standardized spectrophotometrically at 340nm by monitoring NADH production in the 2-oxoglutarate dehydrogenase reaction.

## Results and Discussion

*Concentrations of ATP, ADP, NADH, $NAD^+$, acetyl-CoA, CoA and pyruvate dehydrogenase in rat heart mitochondria*

Table 1 shows concentrations of ATP, ADP, NADH, $NAD^+$, acetyl-CoA and CoA in heart mitochondria from normal rats incubated under conditions that resulted in isolated variations in the ratios of [NADH]/[$NAD^+$] or [acetyl-CoA]/[CoA]. The concentration ratios derived from these data are shown in Table 3. Table 2 compares concentrations of the same metabolites in heart mitochondria of normal and diabetic rats and Table 4 shows concentration ratios derived from these data.

The concentrations of (ATP+ADP), (NADH+$NAD^+$) and of (acetyl-CoA+CoA) in heart mitochondria from normal rats incubated with respiratory substrates averaged 6.4, 6.2 and 1.1nmol/mg of mitochondrial protein respectively. It is assumed that these are intramitochondrial concentrations, as less than 5% was detected in the supernatant after removal of mitochondria by centrifugation (results not shown). The concentration of (ATP+ADP) is somewhat lower (approx. 30%) and that of (NADH+$NAD^+$) somewhat higher than values given by La Noue *et al.* (1973). The concentration of (acetyl-CoA+CoA) was very similar. When heart mitochondria were incubated without substrate the concentration of (ATP+ADP) fell to 3.6nmol/mg of mitochondrial protein. The heart mitochondria used in the present studies were washed rather extensively to remove respiratory substrate, and this could be one factor that determines the concentration of adenine nucleotides. The concentration ratio of ATP/ADP in the present study averaged 3.34. This is somewhat lower than values obtained by La Noue *et al.* (1973), but the concentration of $P_i$ was much lower in the present study (5 as opposed to 20mM). The respiratory-control ratio (Chappell & Hansford, 1965) of heart mitochondria from normal rats prepared by the technique used in the present study varied between 5 and 12.

Heart mitochondria from diabetic rats (Tables 2 and 4) showed lower average concentrations of (ATP+ADP), (NADH+$NAD^+$) and (acetyl-CoA+CoA), the values being 3.1, 4.6 and 0.83nmol/mg of mitochondrial protein. The reason for this has not been ascertained, but the lower values may reflect losses during isolation. Heart mitochondria from diabetic rats are less well coupled than mitochondria from non-diabetic animals. They have a lower respiratory-control ratio and higher matrix water volume (Kerbey *et al.*, 1976), and the ratios of [ATP]/[ADP] and of [NADH]/[$NAD^+$] are lower when incubated with respiratory substrates (see Table 4). An additional factor that may contribute to the lower concentration of (ATP+ADP) is the lower concentration of adenine nucleotides in hearts of diabetic rats (Newsholme & Randle, 1964). However, the concentrations of (NADH+$NAD^+$) and of acid-soluble and acid-insoluble CoA are not decreased in hearts of diabetic rats (Watts, 1969; Garland & Randle, 1964*b*).

The concentration of pyruvate dehydrogenase in heart mitochondria is shown in Tables 3 and 4. Incubation of heart mitochondria for 5min in the absence

Table 1. *Effects of respiratory substrates on concentrations of ATP, ADP, NADH, NAD$^+$, acetyl-CoA and CoA in rat heart mitochondria*

Mitochondria were prepared from hearts of normal rats (see the Experimental section) and incubated at 30°C for the time shown in KCl medium (125mM-KCl/20mM-Tris/Cl/5mM-potassium phosphate/2mM-EGTA, pH7.4) (1mg of mitochondrial protein in 0.5ml) with other additions as shown. For assay of NADH, incubation was terminated with 0.3ml of 1M-KOH in ethanol; for other assays incubation was terminated with 5$\mu$l of 70% (v/v) $HClO_4$. ATP and ADP were assayed with ATP-dependent luciferase; NADH with NADH-dependent luciferase; NAD$^+$, CoA and acetyl-CoA were assayed as NADH with NADH-dependent luciferase (for details see the Experimental section). The same batch of mitochondria was used in experiments with (*a*) 0.4 and 1mM-succinate, (*b*) 2-oxoglutarate plus malate and palmitoylcarnitine at comparable times of incubation and (*c*) 0.4mM-succinate and 0.4mM-succinate+rotenone. No-substrate incubations were made with each batch of mitochondria. Results are means±S.E.M. for the numbers of observations given in parentheses. *$P<0.01$ for (*a*) 0.4mM- versus 1mM-succinate, (*b*) 2-oxoglutarate+malate versus palmitoyl carnitine, (*c*) 0.4mM-succinate versus 0.4mM-succinate+rotenone. For other differences between these pairs, $P>0.05$.

| Mitochondrial incubation | Time of incubation (min) | Metabolite concentrations (nmol/mg of mitochondrial protein) | | | | | |
|---|---|---|---|---|---|---|---|
| | | ATP | ADP | NADH | NAD$^+$ | Acetyl-CoA | CoA |
| No-substrate control | 5 | 1.28±0.01 (39) | 2.30±0.04 (39) | 0.06±0.01 (37) | 5.00±0.05 (24) | 0.13±0.01 (25) | 1.17±0.03 (25) |
| 0.4mM-Succinate | 5 | 5.30±0.07 (8) | 1.60±0.09 (8) | 0.70±0.07 (6) | 4.78±0.02 (6) | 0.02±0.02 (6) | 0.46±0.003 (6) |
| 1mM-Succinate | 5 | 4.90±0.28 (8) | 1.70±0.08 (8) | 2.86±0.08 (6)* | 2.57±0.03 (6)* | 0.06±0.00 (6) | 0.58±0.00 (6)* |
| 250$\mu$M-2-Oxoglutarate+50$\mu$M-L-malate | 2 | 5.13±0.07 (8) | 2.19±0.03 (8) | 0.64±0.01 (4) | 3.93±0.10 (4) | 0.09±0.04 (2) | 0.84±0.00 (2) |
| | 3.5 | 5.01±0.04 (18) | 1.55±0.07 (18) | 1.00±0.03 (8) | 4.40±0.05 (8) | 0.02±0.02 (2) | 0.68±0.02 (2) |
| | 5 | 4.80±0.03 (34) | 1.62±0.04 (34) | 0.65±0.02 (24) | 4.53±0.11 (20) | 0.07±0.01 (9) | 0.40±0.01 (9) |
| 50$\mu$M-Palmitoyl-L-carnitine | 2 | 5.04±0.07 (7) | 1.59±0.13 (4) | 0.51±0.03 (7) | 4.42±0.29 (4) | 0.74±0.00 (2)* | 0.35±0.00 (2)* |
| | 3.5 | 4.95±0.12 (8) | 1.57±0.10 (8) | 0.98±0.03 (8) | 4.86±0.12 (8) | 0.72±0.02 (5)* | 0.30±0.02 (5)* |
| | 5 | 4.36±0.03 (38)* | 1.83±0.06 (36) | 0.50±0.01 (38) | 5.08±0.04 (16)* | 0.74±0.02 (23)* | 0.27±0.01 (23)* |
| 0.4mM-Succinate | 3.5 | 5.25±0.02 (9) | 1.35±0.03 (9) | 2.27±0.01 (3) | 6.54±0.19 (3) | 0.14±0.04 (6) | 1.47±0.07 (6) |
| | 5 | 4.29±0.01 (20) | 1.22±0.01 (20) | 0.76±0.02 (14) | 5.49±0.04 (14) | 0.03±0.03 (6) | 0.77±0.03 (6) |
| 0.4mM-Succinate+rotenone (1.5$\mu$g/ml) | 3.5 | 5.26±0.02 (9) | 1.34±0.03 (9) | 6.78±0.04 (3)* | 2.78±0.09 (3)* | 0.00±0.00 (6) | 2.40±0.06 (6)* |
| | 5 | 3.68±0.02 (12)* | 1.38±0.03 (12)* | 6.74±0.03 (6)* | 1.77±0.11 (6)* | 0.01±0.01 (10) | 1.68±0.04 (10)* |

Table 2. *Concentrations of ATP, ADP, NADH, NAD$^+$, acetyl-CoA and CoA in mitochondria from hearts of normal or alloxan-diabetic rats*

For details of preparation of alloxan-diabetic rats see the Experimental section. Details of preparation and incubation of mitochondria and assay of metabolites are given in the legend of Table 1. Results are means±S.E.M. for the numbers of incubations given in parentheses. *$P<0.01$ for diabetic versus non-diabetic; for other differences between diabetic and non-diabetic, $P>0.05$.

| Mitochondrial incubation | Time of incubation (min) | Metabolite concentrations (nmol/mg of mitochondrial protein) | | | | | |
|---|---|---|---|---|---|---|---|
| | | ATP | ADP | NADH | NAD | Acetyl CoA | CoA |
| No-substrate control | | | | | | | |
| Non-diabetic | 5 | 1.09±0.01 (6) | 2.45±0.03 (6) | 0.06±0.01 (6) | 4.05±0.06 (6) | — | — |
| Diabetic | 5 | 0.79±0.01 (6)* | 1.16±0.03 (6)* | 0.07±0.02 (6) | 3.87±0.06 (6) | — | — |
| 5mM-2-Oxoglutarate+0.5mM-L-malate | | | | | | | |
| Non-diabetic | 3.5 | 6.48±0.31 (3) | 2.18±0.16 (3) | 1.61±0.08 (3) | 4.15±0.06 (3) | 0.06±0.03 (3) | 1.22±0.01 (3) |
| Diabetic | 3.5 | 2.47±0.06 (3)* | 1.13±0.07 (3)* | 0.47±0.03 (3)* | 4.11±0.03 (3) | 0.21±0.13 (3) | 0.92±0.10 (3) |
| 5mM-2-Oxoglutarate+0.5mM-L-malate | | | | | | | |
| Non-diabetic | 5 | 3.39±0.07 (6) | 1.18±0.04 (6) | 1.02±0.09 (6) | 4.86±0.29 (6) | 0.11±0.06 (5) | 0.58±0.02 (5) |
| Diabetic | 5 | 1.88±0.01 (10)* | 0.96±0.03 (10)* | 0.85±0.02 (10) | 3.44±0.05 (10)* | 0.12±0.02 (6) | 0.29±0.02 (6)* |
| 5mM-2-Oxoglutarate+0.5mM-L-malate+0.5mM-pyruvate | | | | | | | |
| Non-diabetic | 3.5 | 6.11±0.18 (3) | 1.66±0.08 (3) | 2.72±0.08 (3) | 2.89±0.12 (3) | 0.02±0.02 (3) | 1.54±0.07 (3) |
| Diabetic | 3.5 | 2.41±0.07 (3)* | 1.06±0.03 (3)* | 0.71±0.01 (3)* | 4.01±0.10 (3)* | 0.02±0.026 (3) | 1.18±0.08 (3) |
| 5mM-2-Oxoglutarate+0.5mM-L-malate+0.5mM-pyruvate | | | | | | | |
| Non-diabetic | 5 | 3.23±0.60 (6) | 1.01±0.05 (6) | 2.55±0.10 (6) | 2.95±0.03 (6) | 0.14±0.02 (5) | 0.84±0.04 (5) |
| Diabetic | 5 | 1.45±0.05 (6)* | 0.84±0.01 (6) | 0.85±0.05 (6)* | 3.96±0.04 (6)* | 0.10±0.02 (6) | 0.49±0.01 (6)* |

Table 3. *Effects of respiratory substrates on concentration of pyruvate dehydrogenase (active form) and ratios of [ATP]/[ADP], [NADH]/[NAD$^+$] and [acetyl-CoA]/[CoA] in rat heart mitochondria*

For details of incubation and assay of metabolites in mitochondria see Table 1. For assay of pyruvate dehydrogenase, incubation mixtures were centrifuged for 20s in an Eppendorff 3200 centrifuge, the supernatant was aspirated and the pellets were frozen in liquid nitrogen. Pyruvate dehydrogenase was extracted at −10°C and assayed spectrophotometrically by coupling to arylamine acetyltransferase (see the Experimental section). *$P<0.01$ for (*a*) 0.4mM-succinate versus 1mM-succinate, (*b*) for 2-oxoglutarate+malate versus palmitoyl-carnitine, (*c*) for 0.4mM-succinate versus 0.4mM-succinate+rotenone. For other differences between these pairs $P>0.01$. Results are means±S.E.M. for the numbers of observations given in parentheses.

| Expt. no. | Mitochondrial incubation | Time of incubation (min) | Pyruvate dehydrogenase activity (munits/mg of mitochondrial protein) | Concentration ratios [Acetyl-CoA]/[CoA] | [ATP]/[ADP] | [NADH]/[NAD$^+$] |
|---|---|---|---|---|---|---|
| — | No-substrate control | 5 | 78.3±1.1 (41) | 0.12±0.01 (25) | 0.67±0.02 (39) | 0.01±0.004 (24) |
| 1 | 0.4mM-Succinate | 5 | 22.3±1.8 (6) | 0.09±0.01 (6) | 3.78±0.32 (8) | 0.14±0.01 (6) |
| 1 | 1mM-Succinate | 5 | 7.9±0.9 (6)* | 0.11±0.03 (6) | 3.61±0.06 (8) | 1.14±0.03 (6)* |
| 2(*a*) | 250μM-2-oxoglutarate | 2 | 34.9±1.3 (8) | 0.11±0.05 (2) | 2.42±0.05 (8) | 0.17±0.01 (4) |
| 2(*b*) | +50μM-L-malate | 3.5 | 43.8±1.6 (13) | 0.03±0.03 (2) | 3.45±0.07 (18) | 0.22±0.01 (8) |
| 2(*c*) | | 5 | 55.6±1.1 (32) | 0.15±0.01 (9) | 3.18±0.10 (34) | 0.16±0.01 (20) |
| 2(*a*) | 50μM-Palmitoyl-L- | 2 | 26.8±0.4 (4)* | 2.11±0.00 (2)* | 3.02±0.27 (4) | 0.16±0.01 (4) |
| 2(*b*) | carnitine | 3.5 | 25.6±0.7 (7)* | 2.44±0.24 (5)* | 3.67±0.52 (8) | 0.20±0.01 (8) |
| 2(*c*) | | 5 | 28.7±0.9 (31)* | 2.79±0.11 (23)* | 2.54±0.12 (36)* | 0.15±0.01 (16) |
| 3 | 0.4mM-Succinate | 3.5 | 23.8±1.9 (6) | 0.22±0.10 (6) | 3.96±0.08 (9) | 0.35±0.01 (3) |
| 3 | | 5 | 30.5±1.4 (12) | 0.07±0.06 (6) | 3.62±0.10 (20) | 0.13±0.001 (14) |
| 3 | 0.4mM-Succinate+ | 3.5 | 16.8±1.2 (6)* | 0.00±0.00 (6) | 4.10±0.11 (9) | 2.47±0.09 (3)* |
| 3 | rotenone (1.5μg/ml) | 5 | 16.4±0.9 (9)* | 0.01±0.01 (10) | 2.79±0.05 (12)* | 4.73±0.16 (6)* |

Table 4. *Effect of alloxan-diabetes on percentage of active pyruvate dehydrogenase and ratios of [acetyl-CoA]/[CoA], [ATP]/[ADP] and [NADH]/[NAD$^+$]*

For details of incubation and assay of metabolites see Table 1. For details of assay of pyruvate dehydrogenase (active form) see Table 3. Total pyruvate dehydrogenase (active+inactive forms) was assayed after incubation with pyruvate dehydrogenase phosphate phosphatase (see the Experimental section). *$P<0.01$ for diabetic versus non-diabetic: † $0.05>P>0.01$ for diabetic versus non-diabetic. For other differences between diabetic and non-diabetic, $P>0.05$. Results are means±S.E.M. for the numbers of incubations given in parentheses.

| Mitochondrial incubation | Time of incubation (min) | Pyruvate dehydrogenase activity (% of total pyruvate dehydrogenase activity) | Concentration ratios [Acetyl-CoA]/[CoA] | [ATP]/[ADP] | [NADH]/[NAD$^+$] |
|---|---|---|---|---|---|
| 5mM-2-Oxoglutarate+ 0.5mM-L-malate | | | | | |
| Non-diabetic | 3.5 | 12.45±0.33 (3) | 0.05±0.02 (3) | 3.41±0.31 (3) | 0.39±0.01 (3) |
| Diabetic | 3.5 | 19.11±1.61 (3)* | 0.27±0.18 (3) | 2.28±0.12 (3)* | 0.11±0.002 (3)* |
| 5mM-2-Oxoglutarate+ 0.5mM-L-malate | | | | | |
| Non-diabetic | 5 | 18.80±1.57 (6) | 0.15±0.08 (5) | 3.64±0.26 (6) | 0.22±0.03 (6) |
| Diabetic | 5 | 13.75±0.94 (6)† | 0.57±0.09 (6)* | 2.16±0.06 (10)* | 0.25±0.01 (10) |
| 5mM-2-Oxoglutarate+ 0.5mM-L-malate+0.5mM-pyruvate | | | | | |
| Non-diabetic | 3.5 | 72.16±1.78 (3) | 0.02±0.02 (3) | 3.89±0.34 (3) | 0.96±0.03 (3) |
| Diabetic | 3.5 | 19.56±0.90 (3)* | 0.03±0.03 (3) | 2.31±0.10 (3)* | 0.18±0.004 (3)* |
| 5mM-2-Oxoglutarate+ 0.5 mM-L-malate+0.5mM-pyruvate | | | | | |
| Non-diabetic | 5 | 75.64±5.08 (6) | 0.23±0.04 (5) | 4.30±0.55 (6) | 0.88±0.02 (6) |
| Diabetic | 5 | 18.63±1.57 (6)* | 0.21±0.04 (6) | 1.77±0.08 (6)* | 0.22±0.01 (6)* |

of respiratory substrate results in almost complete conversion of pyruvate dehydrogenase into its active form (Kerbey *et al.*, 1976). No substrate incubation thus provides an estimate of total pyruvate dehydrogenase activity. The total activity of pyruvate dehydrogenase estimated by this method in heart mitochondria from normal rats was 78munits/mg of mitochondrial protein. This is very similar to the value of 75munits/mg of mitochondrial protein obtained by Kerbey *et al.* (1976). When comparing concentrations of pyruvate dehydrogenase in heart mitochondria from normal rats incubated with different respiratory substrates, it was convenient to compare the concentrations of active dehydrogenase without reference to total activity (i.e. the sum of active and inactive forms). Control experiments (results not shown) showed that none of the conditions of incubation affected the total activity of pyruvate dehydrogenase (measured by incubation of mitochondrial extracts with pyruvate dehydrogenase phosphate phosphatase; see Kerbey *et al.*, 1976). In experiments in which mitochondria from control and diabetic animals were compared, total activities were measured in each experiment, and the concentration of active enzyme is given as a percentage of the total concentration (Table 4).

*Correlation of pyruvate dehydrogenase activity (active form) and ratios of [ATP]/[ADP], [NADH]/[NAD$^+$] and [acetyl-CoA]/[CoA] in rat heart mitochondria*

The object of these experiments was to investigate the proportion of active (dephosphorylated) pyruvate dehydrogenase in heart mitochondria in which (*a*) the ratio of [NADH]/[NAD$^+$] was varied at constant ratios of [ATP]/[ADP] and [acetyl-CoA]/[CoA] and (*b*) the ratio of [acetyl-CoA]/[CoA] was varied at constant ratios of [NADH]/[NAD$^+$] and [ATP]/[ADP]. A wide range of different combinations of respiratory substrates and mitochondrial inhibitors was studied. Table 3 records the results of those experiments (few in number) in which these conditions were met. The results for pyruvate dehydrogenase are given in terms of the concentration of active enzyme, rather than as the proportion of active dehydrogenase, as this facilitated statistical analysis. Within any one experiment the total concentration of dehydrogenase (sum of active and inactive forms) was the same in the two experimental conditions (see the preceding section).

Because of the large number of analyses to be performed, the experiments were conducted as follows. Comparisons between substrate pairs at particular times of incubation (Tables 1 and 3) were performed on the same day and with the same batch of mitochondria as shown by the experimental numbers in Table 3. Comparisons between heart mitochondria from normal and diabetic rats with the same substrates were performed on the same day (Tables 2 and 4). Analyses for ATP, ADP and NADH were made on the same day as the incubation. Analyses for acetyl-CoA, CoA and pyruvate dehydrogenase were made on the following day. Control experiments showed that storage did not affect these concentrations.

With mitochondria incubated in 0.4 or 1mM-succinate there was no significant difference in the ratios of [ATP]/[ADP] or of [acetyl-CoA]/[CoA] after 5min of incubation (Table 3). The ratio of [NADH]/[NAD$^+$] was much greater (8-fold) at the higher succinate concentration as a result of reciprocal changes in [NADH] and [NAD$^+$] (Tables 1 and 3). This increase in the ratio [NADH]/[NAD$^+$] at the higher succinate concentration was associated with a significant fall in pyruvate dehydrogenase (active form) to approximately one-third of that seen with 0.4mM-succinate. These results suggest that an increase in the ratio of [NADH]/[NAD$^+$] facilitated the phosphorylation and inactivation of pyruvate dehydrogenase. However, the ratio of [ATP]/[ADP] was higher at 1mM-succinate at earlier times of incubation (2 and 3.5min; results not shown). It seemed essential to find conditions in which comparable ratios of [ATP]/[ADP] were sustained while [NADH]/[NAD$^+$] was varied.

This was achieved with 0.4mM-succinate ± rotenone (1.5$\mu$g/ml) and with preparations of heart mitochondria in which retention of endogenous substrates was induced by a shorter preparation time using only one wash. Rotenone is an inhibitor of NADH oxidase, which allows formation of NADH but not oxidation of NADH in mitochondria with NAD$^+$-linked substrates (Ernster *et al.*, 1963). Rotenone does not affect succinate oxidation, but reduction of NAD$^+$ by reversed electron transport is inhibited (Ernster *et al.*, 1963). In our experiments rotenone increased the ratio of [NADH]/[NAD$^+$] 7-fold after 3.5min and 20-fold after 5min. We attribute this to the metabolism of endogenous substrates for NAD$^+$-linked dehydrogenases, as the increase in [NADH]/[NAD$^+$] ratio with rotenone was less in well-washed mitochondria (results not shown). The ratio of [acetyl-CoA]/[CoA] was not changed significantly by rotenone at 3.5 or 5min of incubation. The ratio [ATP]/[ADP] was unchanged at 3.5min but fell at 5min. This is acceptable, because a decreased ratio of [ATP]/[ADP] would be expected to increase the proportion of active dehydrogenase, through competitive inhibition of the pyruvate dehydrogenase kinase reaction by ADP (Cooper *et al.*, 1974). In the present experiments rotenone decreased the concentration of active dehydrogenase at both time periods (by approx. 30% at 3.5min and by approx. 45% at 5min). These results, given in Table 3, show that an increased ratio of [NADH]/[NAD$^+$] was associated with a diminution in the proportion of active (de-

phosphorylated) pyruvate dehydrogenase in the absence of any increase in the ratio of [ATP]/[ADP] or of [acetyl-CoA]/[CoA].

With 50$\mu$M-palmitoyl-L-carnitine as compared with 250$\mu$M-2-oxoglutarate+50$\mu$M-L-malate, the ratio of [acetyl-CoA]/[CoA] was increased after 2, 3.5 and 5min of incubation (approx. 20-fold, Table 3). The ratio of [NADH]/[$NAD^+$] was not significantly different with the two conditions of incubation at any time of incubation. The ratio of [ATP]/[ADP] was not significantly different at 2 or 3.5min, but was lower in the palmitoyl-L-carnitine group at 5min. This is acceptable for reasons given in the preceding paragraph. The concentration of pyruvate dehydrogenase (active) form was lower in the palmitoyl-L-carnitine group at all time periods. The decrease was by 25% at 2min, by 42% at 3.5min and by 50% at 5min. These results given in Table 3 show that an increased ratio of [acetyl-CoA]/[CoA] was associated with a diminution in the proportion of active (dephosphorylated) pyruvate dehydrogenase in the absence of any increase in the ratio of [ATP]/[ADP] or of [NADH]/[$NAD^+$).

*Correlation between activity of pyruvate dehydrogenase (active form) and ratios of [ATP]/[ADP], [NADH]/[$NAD^+$] and [acetyl-CoA]/[CoA] in heart mitochondria from normal and diabetic rats*

Heart mitochondria from diabetic rats incubated with 2-oxoglutarate plus malate show a lower proportion of active pyruvate dehydrogenase than do heart mitochondria from non-diabetic rats (Kerbey *et al.*, 1976). In heart mitochondria from normal rats, pyruvate in a range of concentrations from 0.25 to 5mM increases the proportion of active dehydrogenase. This is assumed to be due to inhibition of the pyruvate dehydrogenase kinase reaction (Linn *et al.*, 1969*a*,*b*; Cooper *et al.*, 1974). In heart mitochondria from diabetic rats this effect of pyruvate is very markedly decreased (Kerbey *et al.*, 1976). Tables 2 and 4 show the results of similar experiments in which concentrations of ATP, ADP, NADH, $NAD^+$, acetyl-CoA and CoA were also measured. The results of pyruvate dehydrogenase assays in these experiments are given as the percentage of the active form of the enzyme, because two different batches of mitochondria (i.e. diabetic and non-diabetic) were involved in each experiment. The total pyruvate dehydrogenase was measured after conversion of the inactive (phosphorylated) enzyme with pyruvate dehydrogenase phosphate phosphatase. The results are shown in Tables 2 and 4.

The proportion of active pyruvate dehydrogenase was lower (by 27%) in heart mitochondria from diabetic rats as compared with non-diabetic controls after incubation for 5min (but not at 3.5min) in 2-oxoglutarate (5mM)+malate (0.5mM). The ratios of [ATP]/[ADP] and of [NADH]/[$NAD^+$] were lower in mitochondria from diabetic animals by 33 and 72% respectively. It is assumed that this is due to some degree of uncoupling of oxidative phosphorylation. It is known that heart mitochondria from diabetic rats have lower respiratory-control ratios (Kerbey *et al.*, 1976). The ratio of [acetyl-CoA]/[CoA] was, however, increased significantly in mitochondria from diabetic rats (by 280% at 5min). It is therefore difficult to interpret these findings, since an increased ratio of [acetyl-CoA]/[CoA] would be expected to favour phosphorylation and inactivation of pyruvate dehydrogenase, whereas a decreased ratio of [ATP]/[ADP] and of [NADH]/[$NAD^+$] would be expected to have the opposite effect.

With a combination of 5mM-2-oxoglutarate, 0.5mM-L-malate and 0.5mM-pyruvate, unequivocal results were obtained. Pyruvate at 0.5mM produced the expected increase in the proportion of active dehydrogenase in mitochondria from non-diabetic animals, but it had little if any effect in mitochondria from diabetic animals. Relative to the non-diabetic controls, the proportion of active dehydrogenase was decreased in mitochondria from diabetic animals by 79% at 3.5min and by 75% at 5min. The ratios of [ATP]/[ADP] and of [NADH]/[$NAD^+$] were lower in mitochondria from diabetic rats by 41 and 81% respectively at 3.5min and by 59 and 75% respectively at 5min. The ratio of [acetyl-CoA]/[CoA] was not significantly different at 3.5 or 5min. Thus the phosphorylation and inactivation of pyruvate dehydrogenase in heart mitochondria of diabetic rats was facilitated in spite of unfavourable ratios of [ATP]/[ADP] and of [NADH]/[$NAD^+$].

The data in Table 4 also indicate that pyruvate increases the proportion of active dehydrogenase by direct inhibition of the kinase reaction. The effects of pyruvate were not associated with any obvious diminution in the ratios of [ATP]/[ADP], [NADH]/[$NAD^+$] or [acetyl-CoA]/[CoA].

## General Discussion and Conclusions

The proportion of active pyruvate dehydrogenase in rat heart mitochondria is assumed to reflect the relative rates of the pyruvate dehydrogenase kinase and pyruvate dehydrogenase phosphate phosphatase reactions. Studies with the purified bovine or pig heart pyruvate dehydrogenase complexes have shown that the kinase reaction is inhibited by pyruvate, ADP, pyrophosphate compounds, $Ca^{2+}$, $Mg^{2+}$ and CoA, and activated by NADH, acetyl-CoA and acetoin (Linn *et al.*, 1969*a*,*b*; Cooper *et al.*, 1974, 1975; Pettit *et al.*, 1975). $NAD^+$ either inhibits the kinase reaction (bovine enzyme; Pettit *et al.*, 1975) or reverses NADH activation (pig enzyme; Cooper *et al.*, 1975). The phosphatase reaction is activated by $Mg^{2+}$ (Linn *et al.*, 1969*a*,*b*) and by $Ca^{2+}$ (Randle *et al.*, 1974). Information about regulation of kinase

and phosphatase reactions in the rat heart pyruvate dehydrogenase complex is less complete, but it is known that the kinase reaction is inhibited by ADP, by pyruvate and by pyrophosphate compounds. We shall assume for the purposes of discussion that the rat heart kinase and phosphatase reactions are sensitive to the same effectors as the reactions in pig heart. On this basis the phosphorylation and inactivation of pyruvate dehydrogenase in rat heart mitochondria should be facilitated by (*a*) high ratios of [ATP]/[ADP], [NADH]/[$NAD^+$] and [acetyl-CoA]/[CoA], (*b*) a high concentration of acetoin and (*c*) low concentrations of pyruvate, $Mg^{2+}$, $Ca^{2+}$ and pyrophosphate compounds ($PP_i$ and thiamin pyrophosphate).

The present studies have shown that the phosphorylation and inactivation of pyruvate dehydrogenase in rat heart mitochondria is facilitated by increased ratios of either [NADH]/[$NAD^+$] or [acetyl-CoA]/[CoA]. This conclusion is subject to two assumptions. First, it is assumed that the ratios of the total concentrations of ATP/ADP, NADH/$NAD^+$ and acetyl-CoA/CoA reflect the ratios of the free concentrations of these metabolites. Secondly, it is assumed that there are no variations in the concentrations of other potential effectors of the kinase and phosphatase reactions. The substrates that we have used in these studies do not participate in reactions that form $PP_i$ or acetoin, except for palmitoylcarnitine. This compound could give rise to acetoin via $\beta$-oxidation of palmitoyl-CoA and reversal of part of the pyruvate dehydrogenase reaction sequence (Walsh *et al.*, 1976). Attempts to demonstrate effects of added acetoin on the proportion of active pyruvate dehydrogenase in rat heart mitochondria have been unsuccessful (A. L. Kerbey & P. J. Randle, unpublished work). However, it is not known whether acetoin formed within the mitochondrion is effective in this respect. We have no information on mitochondrial concentrations of thiamin pyrophosphate, $Mg^{2+}$ or $Ca^{2+}$.

It may be noted that the association between an increased ratio of [acetyl-CoA]/[CoA] and a decreased concentration of pyruvate dehydrogenase (active form) was shown in these studies at a low ratio of [NADH]/[$NAD^+$]. These are the conditions in which the pig heart pyruvate dehydrogenase kinase reaction is most sensitive to changes in the ratio of [acetyl-CoA]/[CoA] (Cooper *et al.*, 1975). We have attempted to make similar observations at a high ratio of [NADH]/[$NAD^+$], but have been unable to increase the ratio of [acetyl-CoA]/[CoA] under these conditions. To achieve a high [NADH]/[$NAD^+$] ratio it was necessary to include substrates that can give rise to oxaloacetate (i.e. either malate or succinate). With malate or succinate present, palmitoylcarnitine or acetylcarnitine failed to increase the ratio of [acetyl-CoA]/[CoA] significantly.

The proportion of active pyruvate dehydrogenase in heart mitochondria of diabetic rats was very much decreased relative to that of non-diabetic controls on incubation with pyruvate+2-oxoglutarate+malate. This difference was not accompanied by any increase in the ratios of [ATP]/[ADP], [NADH]/[$NAD^+$] or [acetyl-CoA]/[CoA]. On the contrary, the ratios of [ATP]/[ADP] and [NADH]/[$NAD^+$] were decreased in heart mitochondria from diabetic rats. This finding would indicate that some other mechanism is operative in heart mitochondria in diabetes which either activates the kinase reaction, or blocks inhibition of the kinase reaction by pyruvate, or inhibits the phosphatase reaction. The nature of this mechanism has yet to be identified.

In earlier studies it was suggested that the inhibition of pyruvate oxidation in rat heart by the oxidation of fatty acids or ketone bodies is mediated by an increased ratio of [acetyl-CoA]/[CoA], which can increase up to 60-fold (Garland & Randle, 1964*b*; Randle *et al.*, 1970). This conclusion is supported by the present study. Since the ratio of [acetyl-CoA]/[CoA] is also increased in the heart in diabetes, it was suggested that this change in ratio might also be responsible for impaired oxidation of pyruvate in the heart in diabetes (Garland & Randle, 1964*b*). The results of the present study would indicate that an additional mechanism may be operative and they suggest that this might be quantitatively of greater significance, at any rate at high pyruvate concentrations.

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