Mechanism of Activation of Pyruvate Dehydrogenase by Dichloroacetate and other Halogenated Carboxylic Acids

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1. Monochloroacetate, dichloroacetate, trichloroacetate, difluoroacetate, 2-chloropropionate, 2,2'-dichloropropionate and 3-chloropropionate were inhibitors of pig heart pyruvate dehydrogenase kinase. Dichloroacetate was also shown to inhibit rat heart pyruvate dehydrogenase kinase. The inhibition was mainly non-competitive with respect to ATP. The concentration required for 50% inhibition was approx. 100 μ M for the three chloroacetates, difluoroacetate and 2-chloropropionate and 2,2'-dichloropropionate. Dichloroacetamide was not inhibitory. 2. Dichloroacetate had no significant effect on the activity of pyruvate dehydrogenase phosphate phosphatase when this was maximally activated by Ca^{2+} and Mg^{2+} . 3. Dichloroacetate did not increase the catalytic activity of purified pig heart pyruvate dehydrogenase. 4. Dichloroacetate, difluoroacetate, 2-chloropropionate and 2,2'-dichloropropionate increased the proportion of the active (dephosphorylated) form of pyruvate dehydrogenase in rat heart mitochondria with 2-oxoglutarate and malate as respiratory substrates. Similar effects of dichloroacetate were shown with kidney and fat-cell mitochondria. Glyoxylate, monochloroacetate and dichloroacetamide were inactive. 5. Dichloroacetate increased the proportion of active pyruvate dehydrogenase in the perfused rat heart, isolated rat diaphragm and rat epididymal fat-pads. Difluoroacetate and dichloroacetamide were also active in the perfused heart, but glyoxylate, monochloroacetate and trichloroacetate were inactive. 6. Injection of dichloroacetate into rats starved overnight led within 60 min to activation of pyruvate dehydrogenase in extracts from heart, psoas muscle, adipose tissue, kidney and liver. The blood concentration of lactate fell within 15 min to reach a minimum after 60 min. The blood concentration of glucose fell after 90 min and reached a minimum after 120 min. There was no significant change in plasma glycerol concentration. 7. In epididymal fat-pads dichloroacetate inhibited incorporation of ^{14}C from $[\text{U}-^{14}\text{C}]\text{glucose}$, $[\text{U}-^{14}\text{C}]\text{fructose}$ and from $[\text{U}-^{14}\text{C}]\text{lactate}$ into CO_2 and glyceride fatty acid. 8. It is concluded that the inhibition of pyruvate dehydrogenase kinase by dichloroacetate may account for the activation of pyruvate dehydrogenase and pyruvate oxidation which it induces in isolated rat heart and diaphragm muscles, subject to certain assumptions as to the distribution of dichloroacetate across the plasma membrane and the mitochondrial membrane. 9. It is suggested that activation of pyruvate dehydrogenase by dichloroacetate could contribute to its hypoglycaemic effect by interruption of the Cori and alanine cycles. 10. It is suggested that the inhibitory effect of dichloroacetate on fatty acid synthesis in adipose tissue may involve an additional effect or effects of the compound.

Di-isopropylammonium dichloroacetate raises the respiratory quotient (rate of CO_2 formation/rate of O_2 consumption) and lowers blood glucose concentration on injection into alloxan-diabetic rats (Lorini & Ciman, 1962). Experiments *in vitro* with rat heart and rat diaphragm muscles showed that sodium dichloroacetate stimulates oxidation of glucose or pyruvate, particularly when the oxidation of these substrates is inhibited by alloxan-diabetes or by the respiration of fatty acids or ketone bodies. The compound may also activate phosphofructokinase and glycolysis by decreasing tissue citrate and can inhibit the oxidation of ketone bodies, acetate and long-chain

fatty acids (Stacpoole & Felts, 1970, 1971; McAllister & Randle, 1970; McAllister *et al.*, 1973).

A study of the effects of dichloroacetate *in vivo* and *in vitro* on metabolite concentrations in blood, heart and diaphragm suggested that the compound may activate pyruvate dehydrogenase (EC 1.2.4.1). In mammalian tissues, pyruvate dehydrogenase exists in active (dephosphorylated) and inactive (phosphorylated) forms whose interconversion is catalysed by pyruvate dehydrogenase kinase and pyruvate dehydrogenase phosphate phosphatase (Linn *et al.*, 1969). Preliminary experiments showed that sodium dichloroacetate increases the proportion of the active

form of pyruvate dehydrogenase in the perfused rat heart and may inhibit pyruvate dehydrogenase kinase in purified preparations of pig heart pyruvate dehydrogenase (Whitehouse & Randle, 1973). More detailed evidence on the mechanism of action of dichloroacetate and of related halogenated carboxylic acids has now been obtained from experiments *in vivo*, from experiments *in vitro* with rat heart, diaphragm and epididymal fat-pads and with rat heart, rat kidney and epididymal fat-cell mitochondria, and in studies with pig heart pyruvate dehydrogenase kinase and pyruvate dehydrogenase phosphate phosphatase.

Experimental

Materials

Sodium monochloroacetate, dichloroacetic acid and trichloroacetic acid were from British Drug Houses Ltd., Poole, Dorset, U.K.; 2-chloropropionate and 3-chloropropionate were from Kodak Ltd., Kirby, Liverpool, U.K.; difluoroacetate was from Ralph N. Emmanuel Ltd., Wembley, Middx., U.K.; 2,2'-dichloropropionate was from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.; dichloroacetamide was from Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A. Glyoxylate and Firefly Lantern Extract were from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. The sources of other chemicals and biochemicals were as given by Coore *et al.* (1971), Martin *et al.* (1972) and McAllister *et al.* (1973).

Pyruvate dehydrogenase was purified from pig heart by a modification of the polyethylene glycol-fractionation procedure of Linn *et al.* (1972) (see Randle *et al.*, 1974). Pyruvate dehydrogenase [32 P] phosphate was prepared as described by Denton *et al.* (1972). Pyruvate dehydrogenase phosphate phosphatase was partially purified from pig heart as described by Severson *et al.* (1974). Arylamine acetyltransferase (EC 2.3.1.5) was purified from pigeon livers by the method of Tabor *et al.* (1953).

Methods

Tissues: perfusions and incubations. Hearts were from male albino Wistar rats (250–400 g) which had had free access to water and diet 41 B (Oxoid Ltd., London S.E.1, U.K.). The rats were injected intraperitoneally with heparin (100 units) and Nembutal (60 mg/kg) to prevent blood clotting and to induce anaesthesia before removal of the heart. Alloxan-diabetes was induced by injection of alloxan (60 mg/kg body wt.) intravenously under ether anaesthesia 40–48 h before use. Diaphragms were from rats weighing 130–160 g and fat-pads from rats weighing 140–200 g. Hearts were perfused by drip-through, by a cannula inserted into the aorta, at a perfusion pressure of 8.0 kPa. Hemidiaphragms were incubated in

2 ml of medium in 50 ml conical flasks. Fat-pads were incubated in 5 ml of medium in plastic bottles (100 ml). Perfusions and incubations were made in bicarbonate-buffered medium (Krebs & Henseleit, 1932) gassed with $O_2 + CO_2$ (95:5) at 38°C. Times of perfusion or incubation and additions to perfusion or incubation media are given in the text, Tables or Figures.

Mitochondria: preparation and incubation. Rat heart mitochondria were made in either sucrose-mannitol medium {0.21 M-mannitol, 0.07 M-sucrose, 5 mM-Tris-HCl, 1 mM-EGTA [ethanedioxybis(ethylamine)tetra-acetate], 10 mM-GSH, pH 7.4} or sucrose medium (0.25 M-sucrose, 5 mM-Tris-HCl, 2 mM-EDTA, 10 mM-GSH, pH 7.4). In some experiments hearts were freed of blood and softened by perfusion for 5 min with either 0.154 M-KCl containing 2 mM- $KHCO_3$ and 1 mM-EGTA or with sucrose-mannitol medium. In other experiments hearts were cut into four and blood was removed by washing with sucrose medium. The hearts were then finely divided with scissors and disrupted in a manual Teflon-glass Potter-Elvehjem homogenizer at 0°C. In preparations with prior perfusion mitochondria were separated and washed with a low-speed spin (180–350g for 10 min) followed by two high-speed spins (20000–35000g for 1 min). In preparations without prior perfusion mitochondria were separated and washed with a low-speed spin, two high-speed spins, a low-speed spin and a high-speed spin. Resuspension was made by gentle stirring with a cold glass rod. The yield with either method was approx. 5 mg of mitochondrial protein per heart estimated by the method of Gornall *et al.* (1949). The respiratory-control ratio (Chappell & Hansford, 1969) was 5–12 with 0.25 mM-ADP and with malate (0.5 mM) plus 2-oxoglutarate (5 mM), or pyruvate (5 mM), or palmitoyl-L-carnitine (50 μ M), or acetyl-L-carnitine (4 mM). Rat kidney-cortex mitochondria were prepared by a similar method in sucrose medium. Fat-cell mitochondria were prepared by the method of Martin & Denton (1971).

Mitochondria were incubated in KCl medium (0.12 M-KCl, 2 mM-Tris-HCl, 5 mM-potassium phosphate, 1 mM-EGTA, pH 7.4) at 30°C. O_2 consumption was measured at 30°C with a Clark oxygen electrode by using 4 ml of KCl medium and approx. 1–1.5 mg of mitochondrial protein. For assay of pyruvate dehydrogenase or ATP, approx. 0.5 mg of mitochondrial protein was incubated in 0.5 ml of KCl medium at 30°C in plastic Eppendorff centrifuge tubes. Details of additions and times of incubation are given in the text, Figures or Tables.

Extraction and assay of pyruvate dehydrogenase from tissues or mitochondria in experiments *in vitro*. Perfused hearts were frozen on the cannula with a tissue clamp cooled in liquid N_2 (Wollemberger *et al.*, 1960). Diaphragms and fat-pads were frozen by dropping into liquid N_2 . Pyruvate dehydrogenase was extracted from frozen heart and diaphragm by

the method of Whitehouse & Randle (1973) and from fat-pad by the method of Coore *et al.* (1971). Mitochondria were separated from incubation medium by centrifuging for 1 min at 0°C in an Eppendorff 3200 centrifuge. The supernatant was rapidly discarded and the mitochondrial pellet frozen in liquid N₂. It was then thawed and dispersed in 0.5 ml of 0.1 M-potassium phosphate, pH 7, containing 5 mM-EDTA and the resulting extract was frozen and thawed twice. The active form of pyruvate dehydrogenase was assayed spectrophotometrically by coupling to arylamine acetyltransferase as described by Coore *et al.* (1971). Total pyruvate dehydrogenase was assayed in extracts of tissues or mitochondria after incubation of 50 µl of extract with 40 µl of pyruvate dehydrogenase phosphate phosphatase and 10 µl of 0.1 M-MgCl₂-25 mM-CaCl₂ for 10 min at 30°C.

Assay of pyruvate dehydrogenase kinase. Pyruvate dehydrogenase kinase is an intrinsic component of purified pig heart pyruvate dehydrogenase and was assayed by the incorporation of [³²P]phosphate from [γ -³²P]ATP into pyruvate dehydrogenase, or by the disappearance of pyruvate dehydrogenase activity. Incubations (in 20 mM-potassium phosphate containing 2 mM-mercaptoethanol, pH 7; phosphate-mercaptoethanol buffer) were made in a total volume of 100 µl in Beckman Microfuge tubes at 30°C. The concentrations of pyruvate dehydrogenase, ATPMg²⁺ and other additions are given in the text, Tables and Figures. The reaction was started by addition of ATP and terminated after 2 min (in assays with ³²P) by transferring 30 µl to a 1.5 cm square of Whatman 3 MM paper, which was immediately placed in ice-cold 10% (w/v) trichloroacetic acid. The subsequent procedure for measurement of protein-bound radioactivity was as described by Wastila *et al.* (1971). The disappearance of pyruvate dehydrogenase activity was assayed after 2, 5 and 10 min as described by Whitehouse & Randle (1973). The preparations of pig heart pyruvate dehydrogenase used in these studies were substantially free of ATPase (adenosine triphosphatase); less than 2% of added ATP was hydrolysed/min under the conditions of the kinase assay. The pyruvate dehydrogenase phosphate phosphatase activity was less than 2% of the kinase activity in the preparations of pyruvate dehydrogenase used in this study. The incorporation of ³²P into the dehydrogenase was linear for at least 3 min and there was extremely good correlation between ³²P incorporation and disappearance of pyruvate dehydrogenase activity.

Assay of pyruvate dehydrogenase phosphate phosphatase. The activity of pig heart pyruvate dehydrogenase phosphate phosphatase was assayed by the release of [³²P]phosphate from pyruvate dehydrogenase [³²P]phosphate under conditions described by Randle *et al.* (1974).

Experiments in vivo. Male rats deprived of food for

24 h and weighing 250–300 g were anaesthetized with Nembutal (60 mg/kg) by intraperitoneal injection. Blood samples were obtained by amputating the tip of the tail and collected in plastic cups containing 20 µl of 0.5 M-EDTA. The rats were then given by intraperitoneal injection 0.25 ml of either 0.9% NaCl or 5% (w/v) sodium dichloroacetate/100 g. This injection was repeated at 30 and 90 min. Further blood samples were taken 15, 30, 60, 90, 120 and 180 min after the first injection of NaCl or sodium dichloroacetate.

For assays of pyruvate dehydrogenase in tissue samples unanaesthetized rats were injected intraperitoneally with either 0.9% NaCl or 5% sodium dichloroacetate (see above). After 50 min the rats were anaesthetized with Nembutal, and 10 min later both epididymal fat-pads, the right kidney and a sample of liver were successively removed from one series of rats and frozen with a tissue clamp at the temperature of liquid N₂. A sample of psoas muscle and the heart were removed from another series and frozen. The tissue samples were powdered and pyruvate dehydrogenase was extracted with 0.1 M-potassium phosphate-5 mM-EDTA-10 mM-sodium pyruvate, pH 7 (20 ml/g of powder), with a Polytron PT 10 tissue homogenizer (30 s at position 5). The extracts were centrifuged for 2 min in an Eppendorff 3200 centrifuge and pyruvate dehydrogenase was assayed in the supernatant as described above.

Assay of metabolites. Blood samples (0.2 ml) were deproteinized with 0.8 ml of 5% (w/v) HClO₄, and 0.5 ml of the supernatant was neutralized and perchlorate precipitated with 2 M-KOH containing 20 mM-triethanolamine. Incubation media from incubations of adipose tissue were deproteinized in the same way except that the final concentration of HClO₄ was 2%.

Glucose, lactate, pyruvate and glycerol were assayed spectrophotometrically by the methods of Slein (1963), Hohorst (1963), Bücher *et al.* (1963) and Garland & Randle (1962) respectively.

Mitochondria were deproteinized by addition (per 0.5 ml) of 20 µl of 70% (v/v) HClO₄. ATP was assayed by the luciferin-luciferase assay (Stanley & Williams, 1969).

Incorporations of ¹⁴C. After incubation of fat-pads with [U-¹⁴C]glucose, [U-¹⁴C]fructose or L-[U-¹⁴C]-lactate the contents of Marie flasks were acidified by addition of HClO₄ (final concn. 2%, w/v) and CO₂ was collected in ethyl diphenylamine. The fat-pads were removed, gently blotted and dissolved in 12 ml of 30% (w/v) KOH in 50% (v/v) ethanol and glycerides saponified by heating to 80°C for 1 h. The mixture was cooled and 4 ml of 9 M-H₂SO₄ was added and fatty acids were extracted with 3 × 10 ml of light petroleum (b.p. 40–60°C). The extracts were evaporated to dryness and fatty acids taken up in 15 ml of methoxyethanol-toluene-based scintillator. The

ethyldiphenylamine was taken up in toluene-based scintillator. The composition of methoxyethanol-toluene- and toluene-based scintillators were as given by Severson *et al.* (1974).

Results

Experiments with purified pig heart pyruvate dehydrogenase

The purified pig heart pyruvate dehydrogenase used in these studies catalysed the conversion of pyruvate into acetyl-CoA and CO₂ with concomitant reduction of NAD⁺ to NADH. The specific activity of the various preparations used, assayed spectrophotometrically by measuring ΔE_{340} with 1 mM-pyruvate, 0.5 mM-NAD⁺, 0.1 mM-CoA and 1 mM-thiamin pyrophosphate, was approx. 9 units/mg. The term pyruvate dehydrogenase is used to describe the whole complex, which contains as components pyruvate decarboxylase, acetylhydrolypoate acetyltransferase, dihydrolypoate dehydrogenase and pyruvate dehydrogenase kinase. The kinase was not separated from the other components by centrifuging the complex to a pellet at 150 000g for 90 min. The kinase is therefore tightly bound to the complex and independent variation of the concentration of the kinase and its protein substrate was not possible. As prepared by fractional precipitation the complex contained pyruvate dehydrogenase phosphate phosphatase. This was removed by sedimentation at 150 000g for 90 min in the presence of 1 mM-EDTA, when the phosphatase remained in the supernatant and the other components of the complex formed a pellet. EDTA was then removed by one or more centrifugations in EDTA-free phosphate-mercaptoethanol buffer.

The effects of dichloroacetate (0.5 mM) and of 2-chloropropionate (0.5 mM) on the activity of pyruvate dehydrogenase was followed spectrophotometrically by measuring the initial rate of ΔE_{340} with 1 mM-pyruvate, 0.5 mM-NAD⁺, 0.1 mM-CoA, 1 mM-thiamin pyrophosphate in 50 mM-Tris-HCl-1 mM-MgCl₂-0.5 mM-EDTA-2 mM-2-mercaptoethanol, pH 7.8. No effects of dichloroacetate or of 2-chloropropionate were seen. The activities of pyruvate dehydrogenase (preparation PDH 29 were: control 10 ± 0.4 nmol/min; dichloroacetate 10.5 ± 0.3 nmol/min; 2-chloropropionate 11.0 ± 0.4 nmol/min (means $\pm$ S.E.M. for three determinations). When pyruvate dehydrogenase was subjected to end-product inhibition by addition of acetyl-CoA (to 0.16 mM) and NADH (to 12 μ M) dichloroacetate and 2-chloropropionate (0.5 mM) inhibited the dehydrogenase. The rates were: control 4.4 ± 0.07 nmol/min; dichloroacetate 3.4 ± 0.37 nmol/min; 2-chloropropionate 2.8 ± 0.25 nmol/min (means $\pm$ S.E.M. for three determinations).

The activity of pyruvate dehydrogenase kinase was assayed either by the disappearance of pyruvate de-

hydrogenase activity or by the incorporation of ³²P from [γ -³²P]ATP. Control experiments with a number of different preparations of the dehydrogenase have shown that results with the two methods are closely correlated. The data in Table 1 show that dichloroacetate (1 mM) inhibits pyruvate dehydrogenase kinase substantially when kinase activity is assayed by disappearance of pyruvate dehydrogenase activity. This inhibitory effect of dichloroacetate is also seen in the presence of physiological concentrations of pyruvate and ADP, which are known physiological inhibitors of the kinase (Linn *et al.*, 1969; Martin *et al.*, 1972). The results of similar assays given in Fig. 1 show inhibition of the kinase by 100 μ M- and 1 mM-dichloroacetate but no inhibition by 1 mM-glyoxylate.

The remaining studies of kinase activity were made by the radioactivity assay. As shown in Table 2 dichloroacetate (at concentrations from 25–100 μ M) inhibited kinase activity in the absence and in the presence of thiamin pyrophosphate (10 μ M), a known inhibitor of the kinase (Roche & Reed, 1972). The preparation of pyruvate dehydrogenase (PDH 29) used in the present study was free of thiamin pyrophosphate, as shown by its dependence on added co-enzyme for catalytic activity. Fig. 2 shows the effects on kinase activity of various concentrations of monochloroacetate, dichloroacetate, trichloroacetate, difluoroacetate and dichloroacetamide (10 μ M–1 mM). All except dichloroacetamide were inhibitors of the

Table 1. *Effect of dichloroacetate on the rate of phosphorylation of pig heart pyruvate dehydrogenase in the absence and presence of pyruvate or ADP*

Preparation PDH 22 of pig heart pyruvate dehydrogenase (final concn. 5 units/ml) was incubated with 0.1 mM-ATP and 0.1 mM-MgCl₂ in 20 mM-potassium phosphate-2 mM-2-mercaptoethanol at 30°C with other additions as shown below. Samples were removed at 2, 5 and 10 min and assayed for active pyruvate dehydrogenase by measuring the rate of NAD⁺ reduction at 340 nm (Whitehouse & Randle, 1973). The percentage of pyruvate dehydrogenase phosphorylated was calculated from $100 \times (\text{activity at zero time} - \text{activity at given time}) / (\text{activity at zero time})$. Control incubations in the absence of ATP showed no loss of activity.

Additions	Pyruvate dehydrogenase inactivated by phosphorylation (%) at:		
	2 min	5 min	10 min
None (control)	41	63	81
Pyruvate (50 μ M)	24	38	66
Dichloroacetate (1 mM)	30	32	35
Pyruvate + dichloroacetate	10	14	12
None (control)	46	80	87
ADP (100 μ M)	39	70	80
Dichloroacetate (1 mM)	31	55	61
ADP + dichloroacetate	17	37	47

kinase. The concentration required for half-maximum inhibition of initial rates of phosphorylation was approx. 0.1 mM for monochloroacetate, dichloroacetate and difluoroacetate. Fig. 3 shows the inhibitory effects of the chloropropionates, the relative potencies being 2-chloropropionate > 2,2'-dichloropropionate > 3-chloropropionate. The concentration of 2-chloropropionate required for half-maximum inhibition was approx. 40 μ M.

The inhibition of pig heart pyruvate dehydrogenase kinase by dichloroacetate was found to be mainly non-competitive with respect to ATP by radioactivity assay. In the absence of dichloroacetate the K_m for ATP was $37 \pm 10.0 \mu$ M and the V_{max} (in nmol of protein *P* formed/min per ml) was 1.3 ± 0.12 . In the presence of 0.75 mM-dichloroacetate the K_m for ATP was $13.7 \pm 4.6 \mu$ M and the V_{max} 0.44 ± 0.033 (all values means $\pm$ S.E.M.). The concentrations of ATP were 12.5, 20, 30, 50, 100 and 200 μ M and the specific radioactivity was 50 μ Ci/ μ mol; the concentration of $MgCl_2$ was fixed at 400 μ M; the concentration of pyruvate dehydrogenase was 1.5 units/ml (0.17 mg/ml). Five measurements were made at each ATP concentration.

The effect of dichloroacetate on pig heart pyruvate dehydrogenase phosphate phosphatase was investigated by using pig heart pyruvate dehydrogenase [32 P]phosphate with 32 mM- $MgCl_2$ and Ca^{2+} -EGTA buffer (10 mM-EGTA-9.75 mM- $CaCl_2$; computed free Ca^{2+} 10 μ M). No significant change in phosphatase activity was detected with either 0.1 or 1 mM-dichloroacetate.

Experiments with partially purified rat heart pyruvate dehydrogenase

Pyruvate dehydrogenase was partially purified from powdered frozen rat hearts by the procedure for partial purification of adipose-tissue pyruvate dehydrogenase phosphate phosphatase given by Severson *et al.* (1974). In the final stage of that procedure (centrifuging at 150000g for 90 min) pyruvate dehydrogenase is separated as a pellet. The pellet was

redissolved in 20 mM-potassium phosphate-2 mM-2-mercaptoethanol, pH 7.0, to give 4 units of pyruvate dehydrogenase activity/ml.

The phosphorylation of rat heart pyruvate dehydrogenase was investigated by the radioactivity assay, by using 100 μ M- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (specific radioactivity 200 μ Ci/ μ mol), 2 mM- $MgCl_2$ and 0.8 unit of pyruvate dehydrogenase/ml. Inhibition of phosphorylation was observed with dichloroacetate; inhibition was 34% at 100 μ M, 58% at 0.5 mM and 65% at 1 mM. The inhibition was mainly non-competitive with respect to ATP. In the absence of dichloroacetate the K_m for ATP was $10.3 \pm 2.7 \mu$ M and the V_{max} 52 ± 2.6 pmol of protein *P* formed/min per ml. In the presence of dichloroacetate (0.5 mM) the K_m for ATP was $22.3 \pm 8 \mu$ M and the V_{max} 31 ± 3.1 pmol of protein *P* formed/min per ml. The concentrations of ATP were 20, 25, 35, 50, 100 and 200 μ M, the concentration of $MgCl_2$ was fixed at 2 mM and the concentration of pyruvate dehydrogenase was 0.8 unit/ml.

Fig. 1. Effects of dichloroacetate and of glyoxylate on the inactivation of pyruvate dehydrogenase by phosphorylation

See the legend to Table 1 for conditions of incubation and assay. Experiments were made with preparation PDH 22. ●, No further addition; ○, +1 mM-dichloroacetate; ▲, +100 μ M-dichloroacetate; △, +1 mM-glyoxylate. Each point is the mean of at least nine determinations.

Table 2. Inhibition of pig heart pyruvate dehydrogenase kinase activity by dichloroacetate in the absence or presence of thiamin pyrophosphate

Preparation PDH 29 of pig heart pyruvate dehydrogenase (final concn. 2 units/ml) was incubated with 0.1 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and 2 mM- $MgCl_2$ and other additions as shown for 2 min at 30°C and assayed for protein-bound radioactivity as described in the Experimental section. The incubation medium was 20 mM-potassium phosphate-2 mM-2-mercaptoethanol, pH 7.0. The reaction was initiated after approx. 10 min preincubation by addition of ATP. Each result is the mean $\pm$ S.E.M. of three determinations.

Concn. of thiamin pyrophosphate (μ M)	Concn. of dichloroacetate (μ M) ...	Pyruvate dehydrogenase [32 P]phosphate formed in 2 min (d.p.m./30 μ l of incubation)			
		0	25	50	100
0		1565 $\pm$ 31	1122 $\pm$ 8	998 $\pm$ 8	789 $\pm$ 42
10		991 $\pm$ 27	740 $\pm$ 35	578 $\pm$ 14	485 $\pm$ 19

Experiments with rat heart and rat kidney mitochondria

In experiments with rat heart mitochondria and the oxygen electrode it was found that dichloroacetate (100 μ M) did not support respiration in the presence or absence of 0.25 mM-ADP (total ADP, 1 μ mol). With 50 μ M-palmitoyl-L-carnitine+25 μ M-L-malate, or 0.5 mM-L-malate+5 mM-2-oxoglutarate, or 0.5 mM-L-malate+5 mM-L-glutamate, or 0.5 mM-L-malate+5 mM-acetyl-L-carnitine, dichloroacetate (100 μ M),

difluoroacetate (100 μ M) or 2-chloropropionate (100 μ M) did not change the rate of respiration with or without 0.25 mM-ADP. Thus in these experiments dichloroacetate, difluoroacetate and 2-chloropropionate did not uncouple oxidative phosphorylation or activate mitochondrial ATPases. These compounds did not inhibit adenine nucleotide transport nor did they interfere with the transport or oxidation of malate, glutamate, 2-oxoglutarate, palmitoylcarnitine or acetylcarnitine. Lastly they did not inhibit the

Fig. 2. Effect of various concentrations of monochloroacetate, dichloroacetate, trichloroacetate, difluoroacetate and dichloroacetamide on the incorporation of 32 P from [γ - 32 P]ATP into pig heart pyruvate dehydrogenase

See the legend to Table 2 for conditions of incubation and assay. Experiments were made with preparations PDH 25 and 27. ●, Monochloroacetate; ○, dichloroacetate; ▲, trichloroacetate; △, difluoroacetate; □, dichloroacetamide. Each point is the mean of at least two determinations.

Fig. 3. Effects of 2-chloropropionate, 2,2'-dichloropropionate and 3-chloropropionate on the incorporation of 32 P from [γ - 32 P]ATP into pig heart pyruvate dehydrogenase

See the legend to Table 2 for conditions of incubation and assay. Experiments were made with preparation PDH 27. ●, 2-Chloropropionate; △, 2,2'-dichloropropionate; ○, 3-chloropropionate.

Table 3. Effects of potassium dichloroacetate and potassium 2-chloropropionate on the activity of pyruvate dehydrogenase in rat heart and rat kidney mitochondria

Rat heart and rat kidney mitochondria were prepared as described in the Experimental section. Mitochondria (approx. 0.5 mg of protein) were incubated for 5 min in 0.12 M-KCl-2 mM-Tris-HCl-5 mM-potassium phosphate-1 mM-EGTA, pH 7.4 (0.5 ml) at 30°C, with other additions as shown. Pyruvate dehydrogenase and ATP were extracted and assayed as described in the Experimental section. Results are means $\pm$ S.E.M. for the numbers of observations in parentheses. * $P < 0.01$ versus appropriate control.

Mitochondria	Additions	% of pyruvate dehydrogenase in active form	ATP concentration (nmol/mg of protein)
Heart	None	68 $\pm$ 2.6 (5)	0.7 $\pm$ 0.01 (4)
	0.5 mM-L-Malate+5 mM-2-oxoglutarate	24 $\pm$ 0.7 (3)*	4.6 $\pm$ 0.13 (6)*
	0.5 mM-L-Malate+5 mM-2-oxoglutarate+50 μ M-dichloroacetate	72 $\pm$ 2.3 (3)*	4.6 $\pm$ 0.12 (6)
	5 mM-Succinate	16 $\pm$ 2.7 (2)*	2.8 $\pm$ 0.06 (2)*
	5 mM-Succinate+50 μ M-dichloroacetate	51 $\pm$ 2.1 (2)*	2.7 $\pm$ 0.10 (2)
	5 mM-Succinate+50 μ M-dichloroacetate+50 μ M-2-chloropropionate	58 $\pm$ 1.0 (2)*	2.7 $\pm$ 0.20 (2)
Kidney	None	72 $\pm$ 1.2 (2)	1.2 $\pm$ 0.1 (2)
	0.5 mM-L-Malate+5 mM-2-oxoglutarate	28 $\pm$ 2.9 (4)*	2.6 $\pm$ 0.02 (4)
	0.5 mM-L-Malate+5 mM-2-oxoglutarate+100 μ M-dichloroacetate	73 $\pm$ 0.13 (4)*	2.5 $\pm$ 0.06 (4)

Fig. 4. Activation of pyruvate dehydrogenase in rat heart mitochondria by dichloroacetate

For conditions of incubation, extraction and assay see the legend to Table 3. ●, Mitochondria incubated for 10 min without substrate; ○, mitochondria incubated for 10 min with 0.5 mM-L-malate and 5 mM-2-oxoglutarate; ▲, addition after 5 min of 100 μ M-dichloroacetate to mitochondria incubated in 0.5 mM-L-malate and 5 mM-2-oxoglutarate. Each point is the mean of at least two determinations.

mitochondrial activities of carnitine acetyltransferase or carnitine palmitoyltransferase.

When rat heart mitochondria are incubated in the absence of respiratory substrates the ATP concentration is low (0.7 nmol/mg of protein) and approx. 70% of extracted pyruvate dehydrogenase is in the active form (Table 3). Incubation with 2-oxoglutarate+L-malate increases the ATP concentration sixfold and the proportion of active pyruvate dehydrogenase is markedly decreased. This change in pyruvate dehydrogenase activity is prevented by dichloroacetate (50 μ M) without any change in mitochondrial ATP concentration. Qualitatively similar results are seen with rat kidney mitochondria (Table 3) and rat fat-cell mitochondria (R. M. Denton, personal communication). In rat heart mitochondria 5 mM-succinate produces a somewhat smaller increase in ATP concentration (fourfold) but a larger fall in pyruvate dehydrogenase activity than 2-oxoglutarate+malate. This inactivation of pyruvate dehydrogenase is prevented by 50 μ M-dichloroacetate or 50 μ M-2-chloropropionate (Table 3). Fig. 4 shows that addition of dichloroacetate after 5 min of incubation of rat heart mitochondria with 2-oxoglutarate+malate leads to

Fig. 5. Effect of monochloroacetate, dichloroacetate, difluoroacetate, 2-chloropropionate, 3-chloropropionate and 2,2'-dichloropropionate on pyruvate dehydrogenase activity in rat heart mitochondria

See the legend to Table 3 for conditions of incubation, extraction and assay. The time of incubation was 5 min. Δ , Monochloroacetate; ●, dichloroacetate; ○, difluoroacetate; ▲, 2-chloropropionate; ■, 2,2'-dichloropropionate; □, 3-chloropropionate.

complete re-activation of pyruvate dehydrogenase. These results suggest that mitochondrial oxidation of substrates such as succinate or oxoglutarate+malate (or palmitoylcarnitine+malate; or acetylcarnitine+malate; or glutamate with or without malate; these results are not shown) leads to synthesis of ATP and thus to phosphorylation and inactivation of pyruvate dehydrogenase, and that dichloroacetate and 2-chloropropionate inhibit the phosphorylation and inactivation of pyruvate dehydrogenase.

Fig. 5 shows the effects of various concentrations of monochloroacetate, dichloroacetate, difluoroacetate, 2-chloropropionate, 3-chloropropionate and 2,2'-dichloropropionate on the activity of pyruvate dehydrogenase in rat heart mitochondria incubated with 2-oxoglutarate and L-malate. Monochloroacetate and 3-chloropropionate were relatively ineffective as activators of mitochondrial pyruvate dehydrogenase. The extramitochondrial concentrations for half-maximum activation of the dehydrogenase were approx. 4 μ M (dichloroacetate and 2-chloropropionate), 8 μ M (2,2'-dichloropropionate) and 20 μ M (difluoroacetate).

Effect of dichloroacetate and other halogenated carboxylic acids on pyruvate dehydrogenase activity in perfused rat heart, isolated rat diaphragm and isolated rat epididymal fat-pads

In hearts from non-diabetic rats perfused with medium containing glucose and insulin approx. 20% of pyruvate dehydrogenase was in an active form. The activity observed (5.6 units/g dry wt. at 30°C) may thus be adequate for the rate of pyruvate oxidation in the perfused heart under these conditions

Table 4. *Effects of dichloroacetate, acetate and alloxan-diabetes on pyruvate dehydrogenase activity in perfused rat heart*

Rat hearts were perfused at 38°C for 10 min with bicarbonate-buffered medium (Krebs & Henseleit, 1932) gassed with O₂+CO₂ (95:5) and containing glucose (16.5 mM) and other additions as shown. Where added the concentration of insulin was 20×10^{-3} i.u./ml and that of sodium acetate 5 mM. For details of perfusion and extraction and assay of pyruvate dehydrogenase see the Experimental section. Values for activities are means $\pm$ S.E.M.; the number of hearts perfused is shown in parentheses. ** $P < 0.001$ versus control (no dichloroacetate). † $P < 0.02$ versus non-diabetic heart perfused with medium containing glucose and insulin.

Rat	Addition	Dichloroacetate (mM)	Pyruvate dehydrogenase activity		
			(units/g dry wt.)		(% of active form)
			Active form	Total	
Normal	Insulin	0	5.6 $\pm$ 1.04 (10)	28.2 $\pm$ 0.88 (10)	20
		10	20.4 $\pm$ 2.1 (6)**	23.2 $\pm$ 2.7 (6)	88
		1	23.3 $\pm$ 1.1 (6)**	32.2 $\pm$ 2.2 (4)	73
		0.1	18.7 $\pm$ 0.85 (4)**	27.1 $\pm$ 2.2 (4)	69
		0.01	7.3 $\pm$ 1.17 (4)	22.4 $\pm$ 0.93 (4)	33
Alloxan-diabetic	Insulin	0	1.87 $\pm$ 0.47 (4)†	24.6 $\pm$ 0.93 (4)	8
		10	14.7 $\pm$ 1.3 (4)**	27.3 $\pm$ 2.1 (4)	55
Normal	None	0	1.9 $\pm$ 0.4 (3)	19.4 $\pm$ 1.5 (3)	10
		1	20.5 $\pm$ 1.0 (3)	27.7 $\pm$ 5.4 (3)	73
Normal	Acetate+insulin	0	2.2 $\pm$ 0.29 (8)†	27.2 $\pm$ 2.6 (8)	8
		10	23.2 $\pm$ 1.63 (15)**	31.0 $\pm$ 3.3 (15)	75

Fig. 6. *Reversibility of activation of pyruvate dehydrogenase in perfused rat heart by dichloroacetate*

Hearts were perfused for the times shown with medium containing 16.5 mM-glucose, 5 mM-sodium acetate and insulin (20×10^{-3} i.u./ml). For other details see the legend to Table 2. ●, control; ○, 1 mM-dichloroacetate present throughout; ▲, perfusion switched to medium free of dichloroacetate after 6 min. Each point is the mean of at least two determinations.

(7.2 μ mol/g dry wt. at 38°C; Neely *et al.*, 1972) assuming that the rate of the pyruvate dehydrogenase reaction at 38°C is approximately twice that at 30°C. The proportion of active pyruvate dehydrogenase was substantially increased by dichloroacetate at concentrations between 0.1 and 10 mM. The threshold lay between 10 and 100 μ M and the concentration required for half-maximum activation was approx. 100 μ M. Dichloroacetate was also effective in the

Table 5. *Effect of halogenated carboxylic acids on pyruvate dehydrogenase activity in perfused rat heart*

Rat hearts were perfused for 10 min at 38°C with bicarbonate-buffered medium (Krebs & Henseleit, 1932) gassed with O₂+CO₂ (95:5) containing glucose (16.5 mM) and insulin (20×10^{-3} i.u./ml). For details of perfusion, extraction and assay of pyruvate dehydrogenase see the Experimental section. Results are means $\pm$ S.E.M. of four observations. ** $P < 0.001$ against control. * $P < 0.05$ against control.

Addition	Pyruvate dehydrogenase activity (% in active form)
None (control)	16 $\pm$ 2.2
Monochloroacetate (1 mM)	24 $\pm$ 4.8
Dichloroacetate (1 mM)	66 $\pm$ 5.5**
Trichloroacetate (1 mM)	25 $\pm$ 3.1
None (control)	18 $\pm$ 1.6
Glyoxylate (1 mM)	18 $\pm$ 3.4
Dichloroacetate (1 mM)	61 $\pm$ 6.9**
Dichloroacetamide (1 mM)	39 $\pm$ 7.1*
Difluoroacetate (1 mM)	33 $\pm$ 5.0*

diabetic heart and in the non-diabetic heart perfused with media containing glucose alone or glucose, insulin and acetate. The proportion of the active form of the dehydrogenase was diminished by alloxan-diabetes and by media containing acetate. These results are shown in Table 4. As shown in Fig. 6, the effects of dichloroacetate on pyruvate dehydrogenase activity in perfused rat heart are completely reversed within 6 min of switching to dichloroacetate-free medium. Table 5 shows that monochloroacetate,

trichloroacetate and glyoxylate at 1 mM concentration did not activate pyruvate dehydrogenase in perfused rat heart. Dichloroacetamide (1 mM) and difluoroacetate (1 mM) were active but less effective than the same concentration of dichloroacetate.

As shown in Table 6 dichloroacetate activates pyruvate dehydrogenase in adipose tissue and in diaphragm muscle *in vitro*. In adipose tissue dichloroacetate was as effective as insulin, and neither agent produced any further activation in the presence of the other.

Effect of dichloroacetate on the metabolism of rat epididymal adipose tissue

When insulin activates pyruvate dehydrogenase in adipose tissue the rate of flow of glucose or fructose carbon to glyceride fatty acid and CO₂ is increased. Since dichloroacetate is as effective as insulin in activation of pyruvate dehydrogenase it was decided to investigate its effects on adipose-tissue metabolism.

Table 7 shows that dichloroacetate does not increase the conversion of glucose, fructose or lactate carbon into CO₂ or glyceride fatty acid in spite of

Table 6. *Effect of dichloroacetate on pyruvate dehydrogenase activity in rat diaphragm and rat epididymal fat-pads*

Rat hemidiaphragms and rat epididymal fat-pads were incubated for 1 h at 37°C in bicarbonate-buffered medium (Krebs & Henseleit, 1932), gassed with O₂+CO₂ (95:5). For hemidiaphragms the medium contained glucose (16.5 mM) and insulin (20×10^{-3} i.u./ml). For fat-pads the medium contained fructose (11 mM). Other additions were as shown. Tissues were rapidly blotted and frozen in liquid N₂ and extracted and assayed for pyruvate dehydrogenase as given in the Experimental section. Results are means $\pm$ S.E.M. of four experiments. ** $P < 0.01$ against control. * $P < 0.05$ against control. † $P < 0.05$ against acetate.

Tissue	Addition	Pyruvate dehydrogenase activity		
		(munits/g wet wt.)		(as % of glutamate dehydrogenase activity)
		Active form	Total	
Adipose tissue	None (control)	70 $\pm$ 5	—	16 $\pm$ 0.8
	Dichloroacetate (1 mM)	210 $\pm$ 26**	—	45 $\pm$ 6.0**
	Insulin (2×10^{-3} i.u./ml)	213 $\pm$ 17**	—	38 $\pm$ 6.5**
	Dichloroacetate+insulin	250 $\pm$ 40**	—	47 $\pm$ 4.4**
Diaphragm	None (control)	360 $\pm$ 110	1500 $\pm$ 150	—
	Acetate (5 mM)	290 $\pm$ 10	1600 $\pm$ 150	—
	Dichloroacetate (1 mM)	860 $\pm$ 90*	1400 $\pm$ 300	—
	Acetate+dichloroacetate	700 $\pm$ 150†	1400 $\pm$ 200	—

Table 7. *Effect of sodium dichloroacetate (1 mM) on flow of glucose, fructose or lactate carbon to CO₂ and glyceride fatty acids, and on outputs of pyruvate, lactate and glycerol by rat epididymal adipose tissue*

Rat epididymal fat-pads (two per flask) were weighed and preincubated for 30 min in substrate-free medium and then incubated for 1 h in 5 ml of medium containing [U-¹⁴C]glucose (10 mM; 5 nCi/ μ mol) or [U-¹⁴C]fructose (10 mM; 5 nCi/ μ mol) or [U-¹⁴C]lactate (10 mM; 5 nCi/ μ mol) with or without dichloroacetate. Analyses were then made for ¹⁴CO₂ and ¹⁴C in glyceride fatty acids, and for pyruvate, lactate and glycerol concentrations in the medium as described in the Experimental section. * $P < 0.02$; †, by using paired differences (pads from each rat were assigned to control and dichloroacetate incubations). Results are means $\pm$ S.E.M.; there were five observations in each group.

Substrate	Dichloroacetate (1 mM)	Substrate flow (μ g-atoms of C/h per g of wet pad)		Outputs (μ mol/h per g wet pad)		
		CO ₂	Glyceride fatty acid			
				Pyruvate	Lactate	Glycerol
Glucose (10 mM)	—	3.19 $\pm$ 0.36	3.98 $\pm$ 0.46	0.18 $\pm$ 0.02	0.38 $\pm$ 0.05	0.87 $\pm$ 0.07
	+	3.00 $\pm$ 0.35	3.15 $\pm$ 0.40	0.21 $\pm$ 0.05	0.31 $\pm$ 0.06	0.80 $\pm$ 0.12
Fructose (10 mM)	—	5.89 $\pm$ 0.66	7.61 $\pm$ 1.14	0.30 $\pm$ 0.30	0.45 $\pm$ 0.04	1.01 $\pm$ 0.08
	+	4.98 $\pm$ 0.48	6.08 $\pm$ 0.88*†	0.24 $\pm$ 0.03	0.23 $\pm$ 0.03*	0.8 $\pm$ 0.0*†
L-Lactate (10 mM)	—	6.28 $\pm$ 0.22	4.11 $\pm$ 0.44	0.57 $\pm$ 0.07	—	—
	+	4.69 $\pm$ 0.51*†	2.34 $\pm$ 0.49*†	0.49 $\pm$ 0.01	—	—

Table 8. Effect of sodium dichloroacetate on the outputs of lactate, pyruvate and glycerol, and the flow of fructose or glucose carbon to CO₂, glyceride glycerol and glyceride fatty acids in rat epididymal fat-pads with insulin

For conditions of incubation see the legend to Table 7. Concentrations and specific radioactivities were [U-¹⁴C]-glucose or -fructose (11 mM; 5 nCi/μmol); insulin, 5 × 10⁻³ i.u./ml; sodium dichloroacetate, 1 mM. Values are means ± S.E.M.; there were four observations in each group. * *P* < 0.02.

Substrate	Dichloroacetate	Substrate flow (μg-atoms of C/h per g wet pad)			Outputs (μmol/h per g wet pad)		
		CO ₂	Glyceride glycerol	Glyceride fatty acids	Pyruvate	Lactate	Glycerol
Glucose	—	41.4 ± 1.7	16.2 ± 1.74	48.8 ± 2.2	0.28 ± 0.07	0.77 ± 0.03	—
	+	26.4 ± 1.74*	10.2 ± 0.60*	23.8 ± 4.1*	0.12 ± 0.05	0.27 ± 0.04*	—
Fructose	—	—	—	10.8 ± 0.12	—	0.41 ± 0.07	0.29 ± 0.05
	+	—	—	8.9 ± 0.24*	—	0.25 ± 0.04	0.25 ± 0.03

Fig. 7. Effect of dichloroacetate on blood concentrations of glucose, lactate and glycerol

Rats (250–300 g) were starved overnight and anaesthetized by intraperitoneal injection of Nembutal (6 mg/100 g). Blood samples were collected by amputation of the tail and either 0.9% NaCl (●) or 0.4 M-sodium dichloroacetate (○) was injected intraperitoneally (0.25 ml/100 g body wt.) at 0, 30, and 90 min. Further blood samples were taken at 15, 30, 60, 90, 120 and 180 min. For details of analyses see the Experimental section. There were four rats in each group.

the activation of pyruvate dehydrogenase which it induces. The flow of fructose carbon to glyceride fatty acid was actually lowered by dichloroacetate, and with lactate the flow of carbon to CO₂ and to glyceride fatty acid was lowered. With fructose as

substrate dichloroacetate lowered lactate output and glycerol output significantly.

In the presence of insulin (Table 8), dichloroacetate lowered the flow of glucose carbon to CO₂, glyceride glycerol and glyceride fatty acid, and lowered lactate output. It also lowered the flow of fructose carbon to glyceride fatty acid. Thus the metabolic effects of dichloroacetate in adipose tissue are mainly inhibition of fatty acid synthesis and oxidation of glucose or lactate.

Experiments with dichloroacetate *in vivo*

The data in Fig. 7 show that intraperitoneal injection of dichloroacetate (100 μmol/100 g body wt.) into rats starved overnight lowers the blood concentrations of lactate and glucose but has no effect on that of glycerol. The lactate concentration fell within 15 min and continued to fall to a minimum value, reached at 60 min. Glucose concentration remained steady for 60 min and then fell to reach a minimum value after 120 min. In control rats injected with 0.9% NaCl blood concentrations were essentially unchanged over 180 min.

The activity of pyruvate dehydrogenase was measured in samples of various tissues taken 60 min after the injection of 0.9% NaCl or of dichloroacetate (100 μmol/100 g body wt.). The activities, in units of pyruvate dehydrogenase/g dry wt. of tissue, given as means ± S.E.M. for four observations were (control first): heart, 1.15 ± 0.04, 32.1 ± 0.00 (*P* < 0.001); psoas muscle, 2.0 ± 0.28, 3.4 ± 0.44 (*P* < 0.05); kidney, 1.7 ± 0.09, 4.6 ± 0.36 (*P* < 0.001); liver 2.0 ± 0.11, 2.7 ± 0.17 (*P* < 0.01); epididymal fat-pads, 0.07 ± 0.01, 0.14 ± 0.00 unit/g wet wt. (*P* < 0.001). Thus dichloroacetate produced significant activation of pyruvate dehydrogenase in each of these tissues *in vivo*.

Discussion

Mechanism of activation of pyruvate dehydrogenase by dichloroacetate and other halogenated carboxylic acids

Activation of pyruvate dehydrogenase by dichloroacetate was first suggested by the increase in respiratory quotient in diabetic rats injected with the diisopropylammonium salt (Lorini & Ciman, 1962). Studies *in vitro* with heart and diaphragm muscles showed that sodium dichloroacetate promotes the oxidation of glucose and of pyruvate, and inhibits the oxidations of long-chain fatty acids, 3-hydroxybutyrate and acetate (Stacpoole & Felts, 1970, 1971; McAllister & Randle, 1970; McAllister *et al.*, 1973). Initial consideration was given to the possibility that dichloroacetate may promote the oxidation of pyruvate by inhibition of fatty acid and ketone-body oxidation (Stacpoole & Felts, 1970, 1971). Studies of the effects of dichloroacetate on flow rates in metabolic pathways and on tissue concentrations of metabolites led to the suggestion that dichloroacetate may activate pyruvate dehydrogenase more directly (McAllister *et al.*, 1973). Preliminary observations indicated that dichloroacetate increases the proportion of active (dephosphorylated) pyruvate dehydrogenase in the perfused heart and inhibits the intrinsic kinase of pig heart pyruvate dehydrogenase (Whitehouse & Randle, 1973). This suggestion has been fully confirmed in the present study. Injection of sodium dichloroacetate *in vivo* increased the proportion of active pyruvate dehydrogenase in extracts prepared from heart, psoas muscle, kidney, liver and epididymal adipose tissue. In experiments *in vitro* dichloroacetate increased the proportion of active pyruvate dehydrogenase in perfused rat heart and in isolated rat diaphragm and epididymal fat-pads, and in incubations *in vitro* of mitochondria from rat heart, kidney and epididymal adipose tissue. Attempts to show effects of dichloroacetate in rat liver mitochondria have been frustrated by our inability to extract and assay pyruvate dehydrogenase reliably in these mitochondria.

The mechanism of action of dichloroacetate appears to involve inhibition of pyruvate dehydrogenase kinase, thus facilitating hydrolysis of inactive pyruvate dehydrogenase phosphate to active pyruvate dehydrogenase. The present study has shown that dichloroacetate is an inhibitor of pyruvate dehydrogenase kinase in the pyruvate dehydrogenase complex purified from pig heart and rat heart. The concentration of dichloroacetate required for substantial inhibition of the kinase was in the range 10–250 μM . The concentration required for substantial activation of pyruvate dehydrogenase in rat heart mitochondria was 1–10 μM . The apparently greater sensitivity of rat heart mitochondria may be explained if dichloroacetate is concentrated in intramitochondrial water as are other monocarboxylic acids

(e.g. pyruvate; see Halestrap & Denton, 1974). In the perfused heart the concentration of dichloroacetate required for activation of pyruvate dehydrogenase was 100 μM –1 mM. However, the dichloroacetate space in the perfused heart measured with 10 mM-dichloroacetate was no greater than that of the extracellular marker D-sorbitol (A. McAllister & P. J. Randle, unpublished work). Apparently rat heart muscle is relatively impermeable to dichloroacetate.

The activity of pig heart pyruvate dehydrogenase phosphate phosphatase was not affected by dichloroacetate at saturating concentrations of Ca^{2+} and Mg^{2+} , which are physiological effectors of the enzyme (Severson *et al.*, 1974). These are likely to be the conditions that may occur in the isolated mitochondrion (Severson *et al.*, 1974). Active pyruvate dehydrogenase was not further activated by dichloroacetate, and further, dichloroacetate was a weak inhibitor of pyruvate dehydrogenase in the presence of inhibitory concentrations of acetyl-CoA and NADH. There was no evidence then that dichloroacetate may activate pyruvate dehydrogenase by reversing end-product inhibition *in vivo*. In other experiments (not shown) there was no evidence that dichloroacetate at concentrations up to 1 mM could activate phosphorylated (inactive) pyruvate dehydrogenase. These observations lead us to conclude that dichloroacetate may activate pyruvate dehydrogenase *in vivo* by inhibiting pyruvate dehydrogenase kinase.

Pyruvate dehydrogenase kinase was also inhibited by monochloroacetate, trichloroacetate, difluoroacetate, 2-chloropropionate, 2,2'-dichloropropionate and 3-chloropropionate. Except for monochloroacetate, these compounds also activated pyruvate dehydrogenase in rat heart mitochondria (trichloroacetate was not tested). In the perfused rat heart difluoroacetate activated pyruvate dehydrogenase whereas monochloroacetate and trichloroacetate were inactive. The reason for the inactivity of monochloroacetate in isolated mitochondria and of monochloroacetate and trichloroacetate in the perfused heart is not known. Glyoxylate, which could possibly be formed from dichloroacetate by hydrolysis, did not activate pyruvate dehydrogenase in rat heart mitochondria or perfused rat heart and it was not an inhibitor of pyruvate dehydrogenase kinase. Dichloroacetamide, which had little, if any, inhibitory effect on pyruvate dehydrogenase kinase, activated pyruvate dehydrogenase in the perfused rat heart. It is possible that dichloroacetamide may penetrate cardiac-muscle cells more readily than dichloroacetate and give rise to dichloroacetate within the cell. We have no direct evidence on this point, but the preparation of derivatives of dichloroacetate which are more potent *in vivo* is of practical importance.

The molecular basis of the inhibition of pyruvate dehydrogenase kinase requires further exploration through binding studies. One possibility is that

dichloroacetate may bind to the site at which pyruvate is an inhibitor of the kinase. Data in Table 1 which show further inhibition of the kinase on addition of 50 μ M-pyruvate in the presence of 1 mM-dichloroacetate might not be consistent with this possibility.

Hypoglycaemic action of dichloroacetate

Lorini & Ciman (1962) observed that di-isopropyl-ammonium dichloroacetate is hypoglycaemic in alloxan-diabetic rats but not in non-diabetic rats. Whereas elevation of respiratory quotient was seen in the first hour after injection of dichloroacetate, the fall in blood glucose concentration occurred at a later time. In the present study sodium dichloroacetate was hypoglycaemic in starved non-diabetic rats 90–180 min after the first injection. The hypoglycaemia was preceded by a profound fall in blood lactate concentration. McAllister *et al.* (1973) observed a marked fall in blood lactate and pyruvate concentrations and increased extractions of lactate and pyruvate by the heart in alloxan-diabetic dogs or in non-diabetic dogs receiving Intralipid and heparin by infusion during 60 min after the injection of dichloroacetate. However, no fall in blood glucose concentration was observed during this time. *In vitro*, dichloroacetate stimulated glycolysis and diminished the output of lactate by rat heart and diaphragm muscles. These various observations suggest that dichloroacetate may diminish the

output of lactate by muscle by stimulating pyruvate oxidation and activating pyruvate dehydrogenase. They suggest further that in some tissues (e.g. heart) dichloroacetate may stimulate uptake and oxidation of circulating lactate. These observations suggest one mechanism for the hypoglycaemic effect of dichloroacetate.

As shown in Scheme 1 glucose disappears from the circulation by oxidation or by conversion into fatty acid or glycogen. In starved rats these processes are depressed except for the conversion of glucose into glycogen in cardiac muscle. Glucose is converted into lactate in erythrocytes and in muscle; lactate is released into the circulation and resynthesized into glucose in liver and kidney (the Cori cycle; Cori, 1931). Glucose may also be metabolized to alanine in peripheral tissues by transamination of pyruvate; alanine is released into the circulation and converted into glucose and urea in the liver or kidney (the alanine cycle; Mallett *et al.*, 1969; Felig *et al.*, 1970). The alanine cycle may represent an important mechanism for transfer of amino nitrogen to the liver and for gluconeogenesis. In the starved rat glucose oxidized in the central nervous system may be replaced by gluconeogenesis of glycerol and amino acids. Dichloroacetate, by activating pyruvate dehydrogenase and pyruvate oxidation, may interfere with the release of lactate and alanine from peripheral tissues and impair gluconeogenesis in liver and kidney.

Scheme 1. *Cori and alanine cycles*

In the starved rat, glucose is metabolized by glycolysis (1) in muscle (and erythrocytes) to lactate (2). Alanine is also formed by aminotransferase reactions (3). Pyruvate oxidation (4) is markedly inhibited. Pyruvate, lactate and alanine are transferred through the circulation to liver and resynthesized into glucose (5, 6). In liver metabolism of pyruvate to fatty acid and oxidation of pyruvate are markedly inhibited (4, 7).

Hypoglycaemia could thus result from interruption of the Cori and alanine cycles. Direct evidence for this hypothesis requires measurements of turnover, and information on this point is lacking. The reason for the long latency of the hypoglycaemic effect as compared with the activation of pyruvate dehydrogenase and the fall in blood lactate concentration is not clear. The period of delay (60–90 min) suggests as one possibility adaptive changes in enzyme concentration.

Other metabolic effects of dichloroacetate

McAllister *et al.* (1973) obtained evidence that the action of dichloroacetate in rat heart and rat diaphragm leads to impaired oxidations of acetate, 3-hydroxybutyrate and palmitate, and to a decrease in tissue citrate with consequent activation of phosphofructokinase and glycolysis. McAllister *et al.* (1973) suggested that these effects may be explained by activation of pyruvate oxidation, but direct evidence on this point is not available. The present study has shown that dichloroacetate can inhibit the conversion of glucose, fructose and lactate into glyceride fatty acids and CO₂ in adipose tissue in spite of activation of pyruvate dehydrogenase. This suggests that dichloroacetate may additionally inhibit one or more of the reactions involved in the biosynthesis of fatty acids from glucose, fructose and lactate.

General conclusions

The present study has provided further evidence that dichloroacetate is an inhibitor of pyruvate dehydrogenase kinase and that this may result *in vivo* in activation of pyruvate dehydrogenase and pyruvate oxidation. So far as we are aware, inhibitors of specific regulatory enzymes, which catalyse covalent modification of other enzymes, have not been described previously. The availability of such inhibitors may provide useful information about the biochemical and physiological function of such key regulatory enzymes. Studies of the functions of enzymes and of enzyme regulation in bacteria have been greatly facilitated by the use of mutant strains. The limited occurrence of biochemically useful mutations in animals has been a handicap in studying regulatory mechanisms. Inhibitors, if they can be shown to be specific may be valuable as a means of achieving agenetic mutation. Pyruvate dehydrogenase kinase deficiency has yet to be described, but the present studies suggest that if it occurs, the biochemical disturbances may include low starvation blood concentrations of lactate and glucose.

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