Inhibition of Lactate Gluconeogenesis in Rat Kidney by Dichloroacetate

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1. Sodium dichloroacetate (1 mM) inhibited glucose production from L-lactate in kidney-cortex slices from fed, starved or alloxan-diabetic rats. In general gluconeogenesis from other substrates was not inhibited. 2. Sodium dichloroacetate inhibited glucose production from L-lactate but not from pyruvate in perfused isolated kidneys from normal or alloxan-diabetic rats. 3. Sodium dichloroacetate is an inhibitor of the pyruvate dehydrogenase kinase reaction and it effected conversion of pyruvate dehydrogenase into its active (dephosphorylated) form in kidney *in vivo*. In general, pyruvate dehydrogenase was mainly in the active form in kidneys perfused or incubated with L-lactate and the inhibitory effect of dichloroacetate on glucose production was not dependent on activation of pyruvate dehydrogenase. 4. Balance data from kidney slices showed that dichloroacetate inhibits lactate uptake, glucose and pyruvate production from lactate, but not oxidation of lactate. 5. The mechanism of this effect of dichloroacetate on glucose production from lactate has not been fully defined, but evidence suggests that it may involve a fall in tissue pyruvate concentration and inhibition of pyruvate carboxylation.

Di-isopropylammonium dichloroacetate was shown by Lorini & Ciman (1962) to cause hypoglycaemia in alloxan-diabetic rats. Subsequent investigations showed that sodium dichloroacetate increased the oxidation of glucose and pyruvate and inhibited oxidations of short- and long-chain fatty acids and of ketone bodies in rat heart and diaphragm muscles (Stacpoole & Felts, 1970, 1971; Randle, 1970; McAllister *et al.*, 1973).

Measurement of the changes in tissue-metabolite concentrations with dichloroacetate in perfused heart suggested that the compound may activate the pyruvate dehydrogenase complex (McAllister *et al.*, 1973). Pyruvate dehydrogenase exists in animal tissues in dephosphorylated (active) and phosphorylated (inactive) forms, interconvertible through the pyruvate dehydrogenase kinase and pyruvate dehydrogenase phosphate phosphatase reactions (Linn *et al.*, 1969). Salts of dichloroacetate are inhibitors of the pyruvate dehydrogenase kinase reaction and this action results in conversion of pyruvate dehydrogenase into its active form *in vivo* (Whitehouse & Randle, 1973; Whitehouse *et al.*, 1974). This may result in enhanced oxidation of pyruvate (and hence of lactate and alanine), diminished resynthesis of glucose via the Cori and alanine cycles and hypoglycaemia in starved or diabetic

animals (McAllister *et al.*, 1973; Whitehouse *et al.*, 1974; Blackshear *et al.*, 1974).

Previous studies of the mechanism of action of sodium dichloroacetate have concentrated on its peripheral effects in accelerating the oxidation of pyruvate and of substances that give rise to pyruvate. Since oxidation of pyruvate and glucose formation from pyruvate are potentially competing pathways, we decided to investigate the effects of dichloroacetate on glucose formation from lactate and pyruvate. The inhibitory effect of dichloroacetate on gluconeogenesis from lactate in rat kidney and its mechanism of action is described here.

Experimental

Materials

Chemicals. Dichloroacetic acid from BDH Chemicals, Poole, Dorset, U.K., was redistilled before use. Aqueous solutions were neutralized with NaOH for kidney experiments or use *in vivo* or with KOH for experiments with isolated mitochondria. Biochemical reagents and enzymes were from Boehringer Corp. (London), London W.5, U.K., or Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. Stock solutions of L-lactic acid were standardized by spectrophotometric assay (see under 'Methods') and stored as portions at -10°C . Bovine plasma albumin (fraction V) was defatted by the method of Chen (1967). Albumin was recovered from perfusion media by dialysis against several changes of water at 0°C followed by freeze-drying and treatment with

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acid charcoal as described by Chen (1967). Radioactive compounds were from The Radiochemical Centre, Amersham, Bucks, U.K., except for dichloro[2-³H]acetate, which was synthesized by Mr. P. Crossley from ³H₂O (see Kerbey *et al.*, 1976).

Enzymes. Pyruvate dehydrogenase phosphate phosphatase was partially purified from pig heart (Severson *et al.*, 1974). Arylamine acetyltransferase (EC 2.3.1.5) was purified from acetone-dried pigeon liver powder (Tabor *et al.*, 1953). Pyruvate carboxylase (EC 6.4.1.1) was partially purified from rat liver mitochondria (Chappell & Hansford, 1972) by the method of Scrutton *et al.* (1969) taken to the end of the second (NH₄)₂SO₄ precipitation.

Rats. Male albino Wistar rats were maintained on diet 41B (modified; Oxoid, London S.E.1, U.K.) Alloxan-diabetes was induced by tail-vein injection (60mg/g body wt.) under diethyl ether anaesthesia 48 h before use. Diabetes was confirmed by measuring the blood glucose concentration with Dextrostix (Ames Co., Miles Laboratories, Slough, Bucks, U.K.).

Media. Kidney slices were incubated in bicarbonate-buffered saline gassed at 38°C with O₂/CO₂ (19:1) (Krebs & Henseleit, 1932). Kidneys were perfused with the same medium but with the addition of bovine plasma albumin (70mg/ml) and equilibrated by overnight dialysis against 20vol. of bicarbonate-buffered saline. Mitochondria were incubated at 30°C in KCl medium (120mM-KCl/20mM-Tris/HCl/2mM-EGTA/5mM-potassium phosphate, pH 7.6).

Methods

Kidney-cortex slices. Kidneys from male rats (250g) were bisected, the capsule and medulla removed and slices (5–8mg dry wt.) cut with a Stadie–Riggs microtome. The slices were bisected and duplicate paired halves incubated as test and control in 5ml of bicarbonate-buffered saline at 38°C with shaking. At the end of the incubation, slices were removed, drained on filter paper and either dried at 110°C for dry-weight determination or processed for enzyme or metabolite assays. Media were acidified with 2% (v/v) HClO₄, centrifuged at 0°C for 54000g·min and analysed for metabolites.

Kidney perfusion. This was a modification of the method of Nishiitsutsuji-Uwo *et al.* (1967). A peristaltic pump was used instead of a gravity feed to generate the arterial pressure (Weidemann *et al.*, 1969); the venous and ureteric cannulae were dispensed with, thus shortening the operation time. The arterial cannula was constructed of stainless-steel and not of glass. Male rats (350–450g) were starved overnight and anaesthetized with Nembutal (60mg/g of body wt.) intraperitoneally. The operation proceeded as described by Nishiitsutsuji-Uwo *et al.* (1967) but only ligatures 3, 4 and 5 were required.

After injection of heparin (200 i.u.) into the right ilio-lumbar vein, the arterial cannula was inserted into the mesenteric artery and advanced into the right renal artery and the blood flow replaced immediately by perfusion medium. The kidney was now removed into the perfusion chamber, the medium flowing freely from the open ends of the vena cava. The operation took 5 min to complete and perfusion time was counted from the moment of transfer to the perfusion chamber, when recycling with 100ml of medium began. For monitoring of glucose production, samples (1ml) were taken from the reservoir and added to 0.1ml of 20% (v/v) HClO₄ and stored at –20°C until assayed. The kidney was freeze-clamped at the end of perfusion at liquid-N₂ temperature.

Kidney mitochondria. Mitochondria were prepared from rat kidney cortex by the method of Chappell & Hansford (1972) for preparation of rat liver mitochondria. Mitochondrial protein was measured by the method of Gornall *et al.* (1949). The respiratory-control ratio (Chappell & Hansford, 1972) was 4–6 with 2.5mM-2-oxoglutarate+0.5mM-L-malate.

Pyruvate carboxylation by kidney mitochondria was measured as the incorporation of radioactivity from KH¹⁴CO₃ into acid-stable compounds induced by pyruvate. Mitochondria (0.5mg of protein) were incubated for 5 min at 30°C in sealed vessels in 2ml of KCl medium containing KH¹⁴CO₃ (20mM; 0.1μCi/μmol) adjusted to pH 7.6 and with other additions given in the text or Tables. Reaction was terminated with 0.2ml of 5M-HCl and the contents were taken to dryness at 100°C. The solid residue was taken up in water (0.2ml) and radioactivity assayed by liquid-scintillation spectrometry with toluene/2-methoxy-ethanol-based scintillator (Severson *et al.*, 1974). Corrections were applied for incorporation in blank incubations lacking pyruvate. For assay of mitochondrial ATP, incubations were terminated with 2% (v/v) HClO₄, and KClO₄ and precipitated protein were removed by centrifugation at 0°C (54000g·min).

Assay of metabolites. For the assay of metabolites, frozen kidney was powdered at liquid-N₂ temperature and known weights were extracted with 5% (v/v) HClO₄ (2ml/g of powder). After centrifugation the supernatant was adjusted to pH 7 (glass electrode) with 1M-Tris/HCl, pH 7, containing 5M-KOH, and precipitated⁺KClO₄ was removed at 0°C by centrifugation.

ATP was assayed in HClO₄ extracts of mitochondria or kidney slices without neutralization by the luciferin/luciferase assay method of Stanley & Williams (1969). In neutralized extracts of perfused kidney, or kidney frozen *in vivo*, ATP, ADP, AMP and pyruvate were assayed spectrophotometrically by the methods of Lamprecht & Trautschold (1963) and Adam (1963). Glucose, lactate and pyruvate were assayed in HClO₄ extracts of perfusion or in-

cubation media by the spectrophotometric methods of Huggett & Nixon (1952) and Hohorst (1963). Production of $^{14}\text{CO}_2$ from L-[U- ^{14}C]lactate was measured in sealed vessels by injection of 2% (v/v) HClO_4 into the media and collection in Hyamine injected into a centre well. Radioactivity in Hyamine was assayed by liquid-scintillation spectrometry in a toluene-based scintillator (Severson *et al.*, 1974).

Enzyme assays. Pyruvate dehydrogenase was extracted from frozen kidney powder as described by Whitehouse *et al.* (1974) and the active form assayed spectrophotometrically by coupling to arylamine acetyltransferase (Coore *et al.*, 1971). Total pyruvate dehydrogenase (active+inactive forms) was assayed after conversion on incubation with pyruvate dehydrogenase phosphate phosphatase. Conditions were as given by Whitehouse *et al.* (1974) with addition of 2% (v/v) rat serum (see Wieland, 1975). Pyruvate carboxylase was assayed by two methods. The spectrophotometric assay involved coupling to malate dehydrogenase and measurement of NADH oxidation. The assay buffer was 0.1M-Tris/HCl, pH 7.4, containing 5mM-MgCl₂, 20mM-KHCO₃, 0.2mM-NADH, 0.25mM-CoA, 2mM-acetyl phosphate with malate dehydrogenase (3 units/ml) and phosphotransacetylase (2.5 units/ml). The blank rate caused by any contamination with lactate dehydrogenase was read and pyruvate carboxylation was then initiated with ATP (2mM). The radioactive assay used the same assay buffer with KH $^{14}\text{CO}_3$ (2 $\mu\text{Ci/ml}$), 0.25 unit of phosphotransacetylase/ml and 5 units of citrate synthase/ml. Except where otherwise stated, a unit of enzyme activity is defined as 1 μmol of substrate converted/min. Incubations were for 3 min in sealed bottles and reaction was terminated by injection of 0.2ml of 5M-HCl. After loss of CO_2 , the contents were taken to dryness at 100°C, reconstituted in 0.2ml of water and radioactivity was assayed with toluene/2-methoxyethanol-based scintillator as described above.

Results and Discussion

Effects of dichloroacetate on glucose production by kidney-cortex slices

Table 1 shows rates of glucose production by rat kidney-cortex slices from a number of gluconeogenic substrates. The control rates of glucose production were very similar to those reported by other investigators (e.g., see Krebs *et al.*, 1963), as were the increases in rates associated with starvation or induction of alloxan-diabetes. In the present experiments glucose production from L-lactate or pyruvate was linear with time for at least 90–120min after an initial burst of glucose output (results not shown). Dichloroacetate (1 mM) consistently inhibited glucose production with L-lactate as substrate by approx.

20–30%. Glucose production from lactate with dichloroacetate remained linear and the inhibition only became statistically significant after 60min (results not shown). In general, dichloroacetate had no significant effect on gluconeogenesis rates with substrates other than lactate, although there was some evidence of inhibition ($P<0.05>0.02$) with 2-oxoglutarate (starved for 18h) and with succinate or glutamate (alloxan-diabetic).

The concentration of ATP in kidney slices after 30min of incubation averaged $3.9\pm 0.25\mu\text{mol/g}$ dry wt. (mean $\pm$ s.e.m. of 20 observations) in normal rats and 3.1 ± 0.04 (four observations) in alloxan-diabetic rats. Dichloroacetate (1mM) lowered ATP concentration in slices from normal rats by 19% (lactate as substrate) and by 37% (pyruvate as substrate). Identical changes were seen in slices from alloxan-diabetic rats with lactate as substrate. These changes in ATP concentration were statistically significant ($P<0.01$). It seems unlikely that these changes in ATP concentration were responsible for inhibitory effects of dichloroacetate on glucose production, since the largest fall in ATP concentration was with pyruvate as substrate, but dichloroacetate did not inhibit glucose production from pyruvate (Table 1). This conclusion is supported by the results of experiments with perfused kidney (see below).

Effects of dichloroacetate on glucose production by perfused rat kidney

As judged by flow rate and ATP concentration, the preparation of perfused kidney used in these experiments was satisfactory (see Bowman, 1970; Ross *et al.*, 1973). The mean flow rate with the steel cannula used in the present study was 35ml/min at an arterial pressure of 120mmHg as compared with 24ml/min at 130mmHg with a glass cannula [comparable with rates for glass cannula reported by Ross *et al.* (1973)]. ATP concentration in perfused kidney was approx. 15% lower than in the kidney *in vivo*, but this seemed to reflect a general decrease in the concentration of adenine nucleotides as opposed to a decrease in ratios of ATP/ADP or ATP/AMP (see Table 2). However, there were no significant changes in the concentrations of ATP, ADP or AMP (Table 2) or of total adenine nucleotides (ATP+ADP+AMP, results not shown) or of the concentration ratios ATP/ADP or ATP/AMP (results not shown). Dichloroacetate had no significant effect on individual concentrations (see Table 4) or total concentrations or ratios (results not shown).

Glucose production by perfused kidneys from normal rats was apparently linear with time for 2h with L-lactate or pyruvate as substrate (Fig. 1, Table 3). Rates of gluconeogenesis were therefore calculated

Table 1. *Effect of dichloroacetate on glucose output by rat kidney-cortex slices*

Rat kidney-cortex slices (approx. 10 mg dry wt.) were incubated for 1 h at 37°C in 5 ml of bicarbonate-buffered saline gassed with O₂/CO₂ (19:1) (Krebs & Henseleit, 1932). Substrates were present at 10 mM except for 3-hydroxybutyrate (1 mM), palmitate (0.6 mM) and albumin (1 g/100 ml). At the end of incubation, slices were removed, dried and weighed. The medium was deproteinized with 2% (v/v) HClO₄ and glucose determined spectrophotometrically. For other details see the Experimental section. **P<0.001, *P<0.01, †*P*<0.05; for other differences *P*>0.05 compared with controls. Values are means ± s.e.m. for the numbers of incubations shown in parentheses.

Substrates	Rat	Glucose output (μmol/h per g dry wt.)	
		Control	1 mM-Dichloroacetate
L-Lactate	Normal, fed	89 ± 4.5 (6)	70 ± 3.0 (6) *
L-Lactate, DL-3-hydroxybutyrate		69 ± 1.8 (12)	54 ± 2.4 (12)**
L-Lactate, butyrate		87 ± 2.3 (12)	71 ± 1.2 (12)**
No substrate	Normal, starved for 18 h	27 ± 2.0 (12)	23 ± 3.9 (12)
L-Lactate		116 ± 6.7 (37)	93 ± 5.5 (35)*
L-Lactate, DL-3-hydroxybutyrate		154 ± 12.4 (6)	136 ± 18.8 (6)
L-Lactate, butyrate		119 ± 6.7 (8)	104 ± 9.2 (8)
L-Lactate, albumin, palmitate		131 ± 12.8 (17)	112 ± 4.6 (17)
Pyruvate		173 ± 4.1 (42)	161 ± 4.8 (42)
Alanine		44 ± 0.9 (6)	44 ± 2.1 (6)
2-Oxoglutarate		165 ± 8.2 (9)	138 ± 5.3 (9)†
Glutamate		103 ± 3.4 (12)	101 ± 5.0 (12)
Aspartate		90 ± 3.8 (6)	83 ± 4.2 (6)
Succinate		257 ± 12.3 (6)	252 ± 18.4 (6)
Fumarate		144 ± 11.3 (6)	132 ± 3.5 (6)
Glutamine		68 ± 4.9 (6)	68 ± 3.1 (6)
L-Lactate	Normal, starved for 48 h	137 ± 4.2 (20)	118 ± 3.6 (20)*
L-Lactate	Alloxan-diabetic, fed	172 ± 7.7 (24)	138 ± 7.2 (24)*
Pyruvate		252 ± 11.7 (9)	250 ± 15.3 (9)
Glutamate		129 ± 4.5 (6)	113 ± 4.7 (6)†
Succinate		174 ± 13.6 (6)	139 ± 6 (6)†

Table 2. *Effect of dichloroacetate on adenine nucleotide concentrations in rat kidney*

For conditions of perfusion, see the legend of Table 3; perfusion was for 60 min and dichloroacetate was added after 30 min. In experiments *in vivo*, sodium dichloroacetate was injected intraperitoneally (125 mg/g body wt. at 0 and 30 min) to give a blood concentration of 3.6 mM after 1 h (measured with dichloro[2-³H]acetate). Kidneys were taken for analysis 1 h after the first injection. For other details see the Experimental section. Values are means ± s.e.m. for the numbers of kidneys analysed shown in parentheses. Normal rats were starved for 18 h; diabetic rats were not starved. *P*>0.05 for all differences between control and dichloroacetate.

Experiment	Rats	[Dichloro-acetate] (mM)	Concentration (μmol/g dry wt.)		
			ATP	ADP	AMP
Perfused kidney, 10 mM-L-lactate	Normal	0	5.5 ± 0.73 (8)	2.3 ± 0.24 (8)	0.9 ± 0.06 (6)
		1	4.4 ± 0.30 (6)	2.1 ± 0.12 (6)	0.9 ± 0.10 (6)
Perfused kidney, 10 mM-pyruvate	Normal	0	4.2 ± 0.33 (5)	1.9 ± 0.05 (5)	0.8 ± 0.08 (5)
		1	4.4 ± 0.32 (5)	2.1 ± 0.16 (5)	1.1 ± 0.19 (5)
Perfused kidney, 10 mM-L-lactate	Alloxan-diabetic	0	4.1 ± 0.45 (5)	2.0 ± 0.15 (5)	1.3 ± 0.33 (5)
		1	4.4 ± 0.70 (4)	2.3 ± 0.08 (4)	1.4 ± 0.15 (4)
<i>In vivo</i>	Normal	0	6.5 ± 0.62 (6)	4.0 ± 0.14 (6)	1.7 ± 0.37 (6)
		3.6	7.0 ± 0.24 (6)	3.4 ± 0.19 (6)	1.2 ± 0.08 (6)
<i>In vivo</i>	Alloxan-diabetic	0	5.0 ± 0.31 (3)	2.9 ± 0.40 (3)	1.2 ± 0.32 (3)
		3.6	4.5 ± 0.44 (4)	2.5 ± 0.08 (4)	1.2 ± 0.14 (4)

from the linear regression of glucose production against time for 35–60 and 65–90 min of perfusion. Effects of dichloroacetate were investigated by comparing rates for 35–60 and 65–90 min of

perfusion with addition of dichloroacetate at 60 min (Table 3). Dichloroacetate (1 mM) inhibited glucose production from L-lactate (to 27% of control), but had no effect on glucose production from pyruvate.

Table 3. *Effect of dichloroacetate on glucose output by perfused rat kidney*

Rat kidneys were perfused by recycling with 100 ml of bicarbonate-buffered saline (Krebs & Henseleit, 1932) containing bovine plasma albumin (70mg/ml) through the renal artery at 37°C. Perfusion was for 60 or 120 min and dichloroacetate was introduced at 30 or 60 min respectively. For other details of perfusion and methods of analysis, see the Experimental section. Rates of glucose production were calculated from the linear regression of the mean values for the numbers of perfusion experiments stated; the number of points (glucose production against time of perfusion) is given in parentheses. Values for glucose production are given as the slope $\pm$ s.e. in linear regression analysis. *Comparing 5–30 min periods of perfusion between two groups of diabetic animals, $P>0.05$. **Comparing 35–60 min periods of perfusion between two groups of diabetic animals, $P<0.001$.

Substrate (mM)	Rats	[Dichloro acetate] (mM)	Time (min)	Rates of glucose production (μ mol per min/g dry wt.)	No. of perfusions	<i>P</i>
L-Lactate (10)	Normal	0	35–60	0.72 ± 0.02 (6)	4	<0.001
		0	65–90	0.71 ± 0.04 (6)		
		0	35–60	0.70 ± 0.03 (6)	6	
		1	65–90	0.19 ± 0.04 (6)		
Pyruvate (10)	Normal	0	15–30	5.05 ± 0.19 (6)	4	<0.05
		0	33–48	4.49 ± 0.08 (6)		
		0	15–30	4.70 ± 0.15 (6)		
		1	33–48	4.56 ± 0.26 (6)		
L-Lactate (10)	Alloxan-diabetic	0	5–30	1.49 ± 0.08 (6)	5	<0.001
		0	35–60	0.91 ± 0.03 (6)		
		0	5–30	1.54 ± 0.14 (6)*		
		1	35–60	0.12 ± 0.03 (6)**	4	

Fig. 1. *Glucose production by perfused kidneys from (a) normal rats starved for 18 h and (b) fed alloxan-diabetic rats* Kidneys were perfused by recycling 100 ml of bicarbonate-buffered saline (Krebs & Henseleit, 1932) gassed with O₂/CO₂ (19:1) containing bovine plasma albumin (70mg/ml) and L-lactate (10mM). ●, Control (four perfusions); ▲, dichloroacetate to 1 mM at time shown by arrow (four perfusions). The rates of glucose production in Table 3 (lines 1–4 and 9–12 respectively) were calculated from the slopes of the lines shown in (a) and (b) respectively.

Glucose production by perfused kidneys from alloxan-diabetic rats was not linear (Fig. 1, Table 3). Effects of dichloroacetate on rates of glucose production were measured in two groups of perfused kidneys without or with 1 mM-dichloroacetate added

after 30 min. In the absence of dichloroacetate, rates of glucose production were identical in both groups (5–30 min, Table 3), but addition of dichloroacetate inhibited glucose production to 13% of the control value (35–60 min; Table 3).

Mechanism of action of dichloroacetate on lactate gluconeogenesis in rat kidney

Dichloroacetate inhibits glucose production from lactate, but not from pyruvate in kidney-cortex slices or in perfused rat kidney. Dichloroacetate is known to effect conversion of inactive (phosphorylated) pyruvate dehydrogenase into active (dephosphorylated) pyruvate dehydrogenase, through inhibition of the pyruvate dehydrogenase kinase reaction, in a number of rat tissues including kidney (Whitehouse *et al.*, 1974; see below). In kidney, lactate metabolism is partitioned between oxidation (pyruvate dehydrogenase) and glucose production (pyruvate carboxylase). Thus, if the flux of extracellular lactate to intramitochondrial pyruvate limits overall lactate utilization in rat kidney, activation of pyruvate dehydrogenase and hence pyruvate oxidation with dichloroacetate could inhibit glucose production. This might not obtain with pyruvate as substrate. The K_m of rat kidney pyruvate dehydrogenase for pyruvate is not known. The K_m of bovine kidney and pig heart pyruvate dehydrogenases for pyruvate is 25–50 μM . The K_m of rat kidney pyruvate carboxylase for pyruvate is 240 μM (M. C. Scrutton, personal communication). We have studied the carboxylation of pyruvate in rat kidney mitochondria and the K_m for extramitochondrial pyruvate was 79 μM , which is entirely consistent with Dr. M. C. Scrutton's estimate, as mitochondria concentrate pyruvate (Halestrap & Denton, 1974).

The concentration of pyruvate has been measured in kidneys perfused with 10mM-L-lactate for 60min with or without 1mM-dichloroacetate. With kidneys from normal rats starved for 18h, dichloroacetate lowered the tissue pyruvate from 0.09 ± 0.02 to 0.02 ± 0.008 (mean $\pm$ S.E.M., $\mu\text{mol/ml}$ of tissue water for four observations in each group). With kidneys from fed alloxan-diabetic rats, the values were 0.16 ± 0.03 (control, five observations) and 0.04 ± 0.03 (dichloroacetate-treated, four observations). This fall in tissue pyruvate concentration with dichloroacetate could be an important factor in the observed inhibition of gluconeogenesis; we decided to ascertain whether this was due to activation of pyruvate dehydrogenase and pyruvate oxidation.

Dichloroacetate and pyruvate dehydrogenase activity

The total pyruvate dehydrogenase activity (i.e. the sum of active and inactive forms) averaged 6.8 ± 0.25 units/g dry wt. in kidney slices (mean $\pm$ S.E.M. of 40 observations). The slices were incubated for 60min in medium containing 10mM-lactate or 10mM-2-oxoglutarate with or without 1mM-dichloroacetate. Neither substrate nor dichloroacetate affected total pyruvate dehydrogenase activity. The total activity of pyruvate dehydrogenase in kidneys removed from

fed rats under Nembutal anaesthesia is 6.6 ± 0.71 units/g dry wt. (five observations; Stansbie *et al.*, 1976). The total activity of pyruvate dehydrogenase in kidneys from fed rats perfused for 60min with 10mM-L-lactate was 6.6 ± 0.83 units/g dry wt. (four observations). The concentration of active dehydrogenase and the effect of dichloroacetate is shown in Table 4 (total pyruvate dehydrogenase was not a routine assay in these experiments).

In experiments *in vivo*, the concentration of active dehydrogenase in kidneys from control rats (receiving 0.9% NaCl by injection) was decreased to about 10–15% of the fed normal control by starvation or by alloxan-diabetes, as first shown by Wieland *et al.* (1971). Injection of dichloroacetate increased the concentration of dehydrogenase in kidneys from fed, starved or alloxan-diabetic rats; the increase was about 10-fold in starved or diabetic animals and about 40% in fed normal animals (Table 4). These observations confirm that dichloroacetate effects conversion of pyruvate dehydrogenase into its active form in kidney *in vivo* (cf. Whitehouse *et al.*, 1974).

In kidneys from starved rats or alloxan-diabetic rats perfused with 10mM-L-lactate, the concentration of active dehydrogenase was much higher than in the kidney *in vivo*. This may reflect differences in substrate supply in the two situations. Dichloroacetate (1mM) increased the concentration of active dehydrogenase in kidneys from normal starved rats perfused with 10mM-lactate, but it had no effect with 10mM-pyruvate. The lack of effect with pyruvate was not unexpected because pyruvate is itself an inhibitor of the pyruvate dehydrogenase kinase reactions and may share a common mechanism with dichloroacetate. In kidneys of alloxan-diabetic rats perfused with 10mM-L-lactate for 60min, dichloroacetate did not increase pyruvate dehydrogenase activity (Table 4).

In kidney-cortex slices of fed rats incubated with 10mM-L-lactate or 10mM-2-oxoglutarate, pyruvate dehydrogenase was mainly in the active form (approx. 80% total activity) and dichloroacetate had no effect on the concentration of active dehydrogenase after 60min of incubation. In the same experiment dichloroacetate decreased glucose production with L-lactate from 146 ± 8.3 to 117 ± 7.4 ($P < 0.05$ for 16 observations in each group); and dichloroacetate decreased glucose production with 2-oxoglutarate from 165 ± 8.2 to 138 ± 5.3 ($P < 0.05$ for nine observations in each group).

These observations indicate that the inhibitory effect of dichloroacetate on glucose production from lactate can occur without an increase in the concentration of active pyruvate dehydrogenase. Only in the kidneys of normal starved rats perfused with lactate was the activity of the dehydrogenase increased by dichloroacetate.

Table 4. *Effect of dichloroacetate on activity of pyruvate dehydrogenase (active form) in rat kidney*

In experiments *in vivo*, rats were injected intraperitoneally with dichloroacetate (125 mg/g body wt.) at 0 and 30 min. After 60 min, rats were anaesthetized with Nembutal (see the Experimental section) and the right kidney was removed and freeze-clamped at liquid-N₂ temperature. Kidneys were perfused under conditions given at the head of Table 3 for 60 min and freeze-clamped at liquid-N₂ temperature; dichloroacetate was added after 30 min. Kidney slices were prepared and incubated (see the Experimental section) for 60 min, separated and frozen in liquid N₂. Kidneys or kidney-cortex slices were powdered in a mortar at liquid-N₂ temperature. A weighed amount of powder was extracted in a Polytron PT 10 homogenizer as described by Whitehouse *et al.* (1974) and pyruvate dehydrogenase was assayed spectrophotometrically by coupling to arylamine acetyltransferase as described by Coore *et al.* (1971). The concentration of dichloroacetate was 1 mM in slice and perfusion experiments. The concentration of dichloroacetate in the circulation in experiments *in vivo* was 3.6 M after 60 min. *P < 0.01 compared with control. **P < 0.001 compared with control. Values are means ± S.E.M. for the numbers of observations given in parentheses.

Experiment	Rats	Pyruvate dehydrogenase (active form) (units/g dry wt.)	
		Control	Dichloroacetate
Measurements <i>in vivo</i>	Normal, fed	3.9 ± 0.34 (6)	6.4 ± 0.53 (6)*
	Normal, starved for 48 h	0.4 ± 0.02 (6)	4.8 ± 0.27 (6)**
	Normal, starved for 18 h	0.6 ± 0.06 (6)	5.8 ± 0.43 (6)**
	Alloxan-diabetic, fed	0.4 ± 0.06 (3)	4.2 ± 0.34 (4)**
Perfusion, 10 mM-L-lactate	Normal, starved for 18 h	3.2 ± 0.45 (8)	5.8 ± 0.76 (5)*
Perfusion, 10 mM-pyruvate		4.8 ± 0.87 (5)	4.5 ± 0.39 (5)
Perfusion, 10 mM-L-lactate	Alloxan-diabetic, fed	5.2 ± 0.58 (5)	4.6 ± 0.41 (4)
Slices, 10 mM-L-lactate	Normal, fed	5.8 ± 0.49 (16)	5.4 ± 0.42 (16)
Slices, 10 mM-2-oxoglutarate		5.4 ± 0.35 (4)	5.4 ± 0.43 (4)

Table 5 *Effect of dichloroacetate on metabolism of L-lactate in rat kidney-cortex slices*

Slices (approx. 40 mg dry wt.) from kidney cortex of fed rats were incubated for 60 min in 10 ml of medium containing L-[U-¹⁴C]lactate (10 mM; 2.5 nCi/μmol). The disappearance of lactate and the production of glucose, pyruvate and ¹⁴CO₂ was assayed (see the Experimental section). *P < 0.05 compared with control. **P < 0.01 compared with control for four observations in each group. Values are means ± S.E.M. for four observations.

	Lactate (μmol/g dry wt.)	
	Controls	Dichloroacetate (1 mM)
Lactate in medium	-222.4 ± 4.2	-189.4 ± 5.1**
Lactate appearance as		
glucose	64.7 ± 3.8	46.0 ± 1.2**
CO ₂	112.9 ± 4.0	113.2 ± 4.6
pyruvate	25.6 ± 2.6	13.8 ± 0.4*
	203.2 ± 7.0	173.0 ± 5.7*
Balance (lactate diverted to other products)	19.2 ± 9.1	16.4 ± 4.2

Dichloroacetate and metabolic fate of lactate

Table 5 shows the effects of dichloroacetate (1 mM) on the metabolism of lactate in kidney-cortex slices from fed normal rats incubated for 60 min with [U-¹⁴C]lactate (10 mM; 2.5 nCi/μmol). Dichloroacetate decreased lactate uptake by 33 μmol/g dry wt. (by 16% of control uptake). This difference was

partitioned between glucose production and pyruvate output; the rate of ¹⁴CO₂ production from lactate and the small balance of lactate uptake unaccounted for by glucose, pyruvate and ¹⁴CO₂ production were unaffected by dichloroacetate. The unchanged flux of [U-¹⁴C]lactate to ¹⁴CO₂ may thus be consistent with the lack of activation of pyruvate dehydrogenase noted, and the fall in pyruvate output correlates with the decline in tissue pyruvate given above.

The high activity of pyruvate dehydrogenase in kidney tissue incubated or perfused with lactate may result from partial inhibition of pyruvate dehydrogenase kinase by pyruvate formed from lactate within the tissue. The preservation of pyruvate dehydrogenase activity and of pyruvate oxidation with dichloroacetate may thus involve inhibition of the kinase reaction by dichloroacetate (tissue pyruvate concentration decreased with dichloroacetate).

Dichloroacetate and pyruvate carboxylation in kidney mitochondria

The early reactions in glucose production from lactate involve cell uptake of lactate, the lactate dehydrogenase reaction, mitochondrial uptake of pyruvate and the pyruvate carboxylase reaction. Dichloroacetate inhibited lactate uptake in kidney slices, but whether this was a primary action of dichloroacetate on lactate uptake or secondary to diminished lactate conversion into glucose could not be ascertained. The lactate dehydrogenase reaction in a supernatant fraction of rat kidney cortex was not

Table 6. *Effect of dichloroacetate on pyruvate carboxylation in rat kidney-cortex mitochondria*

Rat kidney mitochondria (0.5 mg of protein) were incubated in 2 ml of KCl medium (120 mM-KCl, 25 mM-Tris/HCl, 5 mM-potassium phosphate, 2 mM-EGTA, pH 7.6) containing $\text{KH}^{14}\text{CO}_3$ (20 mM; $0.1 \mu\text{Ci}/\mu\text{mol}$) adjusted to pH 7.6. Incubations were for 5 min at 30°C in sealed vessels. The reaction was terminated by addition of 0.2 ml of 5 M-HCl, and the contents were taken to dryness at 100°C. The solid residue was taken up in 0.2 ml of water and radioactivity assayed by liquid-scintillation spectrometry with toluene/2-methoxyethanol scintillator (Severson *et al.*, 1974). The incorporations were corrected by blank incubations lacking pyruvate. Values are means $\pm$ S.E.M. for the numbers of incubations shown in parentheses. * $P < 0.01$ compared with control. ** $P < 0.001$ compared with control.

Additions (mM)	[Pyruvate] (mM)	[Dichloroacetate] (mM)	Pyruvate carboxylation (nmol/mg of protein per ml)
None	0.5	0	6.04±0.18 (6)
		2.5	6.00±0.17 (6)
L-Glutamate (2.5) + n-octanoate (0.5)	0.5	0	9.94±1.04 (6)
		2.5	10.22±0.25 (6)
			Pyruvate carboxylation (% of control without dichloroacetate)†
L-Glutamate (5)	0.06	0.01	99±1 (2)
		0.05	106±1 (2)**
		0.20	97±5 (2)
		0.50	99±3 (6)
		1	97±4 (6)
		2	84±2 (4)*
		5	80±1 (3)*
		10	63±1 (4)**

† These results were obtained in a number of separate experiments and are most readily expressed as % of control.

inhibited in either direction by 1 mM-dichloroacetate. The effect of dichloroacetate on the uptake and carboxylation of pyruvate by rat kidney-cortex mitochondria is shown in Table 6. The uptake and carboxylation of pyruvate was measured by the incorporation of radioactivity from $\text{KH}^{14}\text{CO}_3$ into acid-stable compounds.

Preliminary experiments showed that the K_m for pyruvate carboxylation in mitochondria was $79 \mu\text{M}$ for extramitochondrial pyruvate (calculated from a Lineweaver-Burk plot with five pyruvate concentrations between $50 \mu\text{M}$ and 2 mM). As shown in Table 6, 2.5 mM-dichloroacetate had no effect on the carboxylation of pyruvate, with 0.5 mM-pyruvate, even when activated by the presence of glutamate and octanoate. With $60 \mu\text{M}$ -pyruvate, carboxylation was inhibited by 16% at 2 mM-dichloroacetate and by 37% at 10 mM-dichloroacetate. There are three potential mechanisms for this inhibition. Dichloroacetate may enter mitochondria by the pyruvate transporter (Halestrap, 1975) and thus may interfere with pyruvate uptake. Dichloroacetate at 5 mM lowered mitochondrial ATP by 12% (results not shown) and this could inhibit pyruvate carboxylase via an increase in mitochondrial ADP/ATP ratio (Wimhurst & Manchester, 1970). The possibility that dichloroacetate may inhibit pyruvate carboxylase directly was not tested with kidney pyruvate carboxyl-

ase, but dichloroacetate (0.5, 1 and 2 mM) had no effect on the activity of partially purified liver pyruvate carboxylase over a range of pyruvate concentrations from $50 \mu\text{M}$ to 1 mM.

Discussion

When dichloroacetate is given *in vivo* to starved or alloxan-diabetic rats or to diabetic patients, it lowers blood concentrations of lactate, pyruvate, alanine and glucose (McAllister *et al.*, 1973; Blackshear *et al.*, 1974, 1975; Whitehouse *et al.*, 1974). These effects of dichloroacetate have been attributed to conversion of pyruvate dehydrogenase into its active form with concomitant removal of lactate, pyruvate and alanine by oxidation. The fall in blood glucose concentration is assumed to result from this interruption in the normal supply of gluconeogenic precursors. The present study has shown that in the rat kidney glucose production itself is inhibited when the substrate is lactate, with concentrations of dichloroacetate found in the circulation after the administration of effective doses *in vivo*; this contrasts with the lack of effect in rat hepatocytes (Crabb *et al.*, 1976; S. C. Sharples & P. J. Randle, unpublished work). It is therefore possible that this direct inhibitory action in the kidney by dichloroacetate could contribute to its hypoglycaemic effect, though it is

not likely to be a major factor because hypoglycaemia is associated with a marked decrease in plasma concentrations of lactate and pyruvate.

The present study has suggested that much of the inhibitory effect of dichloroacetate on renal lactate gluconeogenesis may be the result of a substantial decrease in tissue pyruvate concentration. The extent of this decrease (75%) is comparable with the degree of gluconeogenesis inhibition. The extent to which this decrease in pyruvate concentration may contribute to the decrease in the rate of gluconeogenesis will depend on the mitochondrial pyruvate concentration relative to the K_m for pyruvate carboxylase. We do not know the mitochondrial pyruvate concentration in kidney; the whole tissue pyruvate concentration in control perfusions (90 μM in kidneys from normal rats) was low in relation to the K_m (240 μM). The present study has also shown that dichloroacetate (2–10 mM) can inhibit pyruvate carboxylation in kidney-cortex mitochondria at low pyruvate concentration (60 μM) but not at high pyruvate (0.5 mM). The extent of this inhibition *in vivo* may therefore depend on cytoplasmic concentrations of dichloroacetate in the kidney. When dichloro[2- ^3H]acetate is injected into rats, the plasma concentration is maintained above 1 mM for several days (J. H. Lacey, S. C. Sharples & P. J. Randle, unpublished work). This suggested active transport of dichloroacetate by renal tubular cells and perfusion studies have confirmed this (S. C. Sharples, B. D. Ross & P. J. Randle, unpublished work). However, measurements of dichloroacetate space in kidney *in vivo* with dichloro[2- ^3H]acetate showed that the concentration of dichloroacetate in intracellular water in the kidney is unlikely to be more than twice that of extracellular water (dichloroacetate space $0.86 \pm 0.01 \text{ ml/g wet wt.}$, mean $\pm$ S.E.M. of three observations; total water space 0.72 ml/g wet wt.). If the intracellular concentration of dichloroacetate is 2 mM with a perfusate concentration of 1 mM, the inhibition of pyruvate carboxylation may only be 16% (Table 6). Thus this effect of dichloroacetate may only contribute modestly to the overall inhibition of lactate gluconeogenesis.

The reason for the fall in tissue pyruvate with dichloroacetate has not been fully defined in the present study. It does not appear to require activation of pyruvate dehydrogenase or enhanced oxidation of pyruvate. It is associated with a diminished uptake of lactate by the kidney. It could be due to inhibition of lactate uptake by dichloroacetate or to an increased cytoplasmic NADH/NAD $^+$ ratio. We have not been able to measure intracellular lactate reliably in perfusions with 10 mM-lactate because of the large contribution of extracellular lactate to the total space. Attempts to measure cytoplasmic NADH/NAD $^+$ by the glycerophosphate dehydrogenase couple were frustrated by the very low tissue con-

centration of dihydroxyacetone phosphate. Dichloroacetate can inhibit the tubular reabsorption of lactate in perfused kidney (B. D. Ross, personal communication) but the effect is too small to account for more than 20% of the inhibition of glucose production. The observation that dichloroacetate is an inhibitor of lactate gluconeogenesis in kidney, but not in liver, and that it is more effective in the perfused kidney than in slices, might suggest nevertheless that the functional topography of the kidney (i.e. glomerular filtration and tubular function) is involved.

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