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PROLONGED HYPOLACTATEMIA AND INCREASED TOTAL PYRUVATE DEHYDROGENASE ACTIVITY BY DICHLOROACETATE

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Abstract—Dichloroacetate (DCA) given gastrically as a single dose to healthy, fed rats caused transient lowering of blood glucose, lactate, and pyruvate. Chronic daily dosing caused lowering of these metabolites and a delay in the return of lactate to basal levels for 48 hr after the final dose. DCA caused activation of the pyruvate dehydrogenase complex (PDHC), with acute multiple dosing or chronic daily dosing. The elevated active PDHC persisted for 12 hr following the final dose. In addition, total PDHC activity was increased with chronic dosing and persisted for 48 hr following the final dose. This increase was not blocked by protein synthesis inhibitors. DCA increased isolated hepatocyte [^{14}C-1]pyruvate oxidation and activated hepatocyte PDHC. Glyoxylate and oxalate, hepatic metabolites of DCA, were inhibitory at similar concentrations.

Sodium dichloroacetate (DCA) reduces circulating levels of lactate, pyruvate and alanine in animals [1, 2] and humans [3, 4] by its action on the pyruvate dehydrogenase complex (PDHC) (EC 1.2.4.1), a mitochondrial enzyme system catalyzing the conversion of pyruvate to acetyl CoA and CO_2. PDHC is regulated, in part, by a phosphorylation–dephosphorylation mechanism [5]. A PDHC kinase converts PDHC to an inactive, phosphorylated form while a specific phosphatase reconstitutes the active enzyme [6]. Noncompetitive inhibition by DCA of PDHC kinase [7, 8] allows unopposed activation of PDHC by the phosphatase and accelerates pyruvate, lactate and alanine flux through the enzyme. Consequently, release of lactate and alanine from peripheral tissues into the circulation is reduced [9] and fewer 3-carbon fragments are available for hepatic glucose synthesis. This mechanism contributes, in part, to the blood glucose-lowering effect of DCA observed in animals [2] and humans [4] in conditions such as starvation or diabetes in which gluconeogenesis is increased.

Rat liver rapidly metabolizes DCA to glyoxylate and oxalate [10], and may account for the short half-life of DCA observed in several species [3, 11]. The effect of DCA on blood lactate, pyruvate, alanine and glucose levels, however, persists hours to days following plasma clearance of the drug [2–4]. This may be due to direct effects of DCA on PDHC levels or to the accumulation of active drug metabolites. Accordingly, the present study was designed to evaluate the mechanism of the prolonged effects of DCA.

We investigated the metabolic effects of DCA in rats *in vivo* and correlated changes in circulating glucose, lactate and pyruvate levels with changes in hepatic *in vitro* PDHC activity. We found that oral DCA treatment did not affect hepatic PDHC synthesis but appeared to stabilize pre-existing enzyme activities and maintain high total PDHC activity *in vivo*. Addition of DCA to suspensions of isolated hepatocytes stimulated [^{14}C-1]pyruvate oxidation and PDHC activity. In contrast, glyoxylate and oxalate inhibited pyruvate oxidation and PDHC activity in hepatocytes.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (225 ± 25 g) were used and had free access to water and Purina chow.

Chemicals

DCA was obtained from the Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan), [^{14}C-1]pyruvate from the New England Nuclear Corp. (Boston, MA), and all other chemicals from the Sigma Chemical Co. (St. Louis, MO).

Liver cell isolation

Isolated, morphologically intact, hepatic parenchymal cells were obtained in high yield by modification of the method of Berry and Friend [12]. The perfusion medium was an oxygenated, calcium-free Krebs–Henseleit original Ringer bicarbonate buffer (pH, 7.4) containing 0.05% collagenase. Isolated cells were obtained as described previously [13]. At least 95% of the final cell preparation obtained from

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each liver consisted of parenchymal cells which excluded trypan blue stain.

Analyses

In vitro *studies*. The isolated hepatocyte incubation medium contained 0.15 M NaCl, 0.15 M KCl, 0.11 M $CaCl_2$, 0.15 M KH_2PO_4, 0.15 M $MgSO_4 \cdot 7H_2O$, 25 mM $NaHCO_3$ and 1.5% bovine serum albumin (pH 7.4). For estimation of [^{14}C-1]pyruvate oxidation, 2×10^5 cells in 0.5 ml medium were incubated in 10 ml plastic scintillation vials with or without added drug in a final volume of 1.0 ml for 30 min at 37° with gentle shaking (~70 rpm); 0.1 mM pyruvate (0.5 μCi [^{14}C-1]pyruvate/ml) was then added to the cell suspension. The vials were fitted with rubber stoppers suspending disposable center wells which contained a small ribbon of filter paper. Cells were incubated with shaking for an additional 5 min. The reaction was stopped by addition of 1.5 ml of 0.8 M citric acid and 0.4 M potassium phosphate (pH 3.0) solution. NaOH (0.2 ml, 1 M) was added to the center wells and the vials were incubated with shaking for an additional 30 min to allow trapping of $^{14}CO_2$. The center wells were then removed, placed in 10 ml ACS scintillation fluid, and counted for 10 min in a Beckman model 1000 counter. The reaction was linear for the number of cells and length of incubation.

For estimation of *in vitro* effects of drugs on PDHC activity, 4–8 $\times 10^6$ hepatocytes were incubated in 25 ml polycarbonate flasks with or without added drug in a final volume of 3.0 ml. The cell suspension was gassed continuously with 95% O_2–5% CO_2 and incubated with shaking for 30 min at 37°. The reaction was stopped by plunging the flasks in ice and rapidly centrifuging the suspension at 5000 *g* for 5 min. The resulting cell pellet was frozen in liquid N_2, stored at −70°, and assayed within 48 hr.

Hepatocyte PDHC activity was determined by modifying the technique of Blass *et al.* for platelet enriched plasma [14] by using 10.0 mM phosphate buffer and shortening the incubation time to 5 min. Tissue PDHC from frozen liver was assayed as previously described [15]. The active PDHC ($PDHC_a$) was assayed immediately following homogenization. Total PDHC activity ($PDHC_t$) was assayed following *in vitro* activation with 2 mM $CaCl_2$ and 20 mM $MgCl_2$. Maximum activation was complete after 60 min of preincubation (data not shown).

Lipoamide dehydrogenase (LAD) (EC 1.6.4.3) activity was estimated by the method of Stumpf and Parks [16]. Pyruvate decarboxylase (PDC) (EC 4.1.1.1) activity was determined after the method of Blass *et al.* [17]. This assay differs from PDHC in that incubation occurs in the absence of cofactors thiamine pyrophosphate and Co-A, at pH 6.0 instead of 7.4, and in the presence of potassium ferricyanide. Pyruvate carboxylase (PC) (EC 6.4.1.1) activity was assayed by the method of Utter and Keech [18]. Protein was determined by the method of Lowry *et al.* [19].

In vivo *studies*. For PDHC experiments, DCA was adminstered by gastric intubation at a dose of 100 mg/kg in a solution of 200 mg/ml saline. Control animals received only saline. For single dose experiments, DCA was administered to rats that were killed at 0.5, 1, 3, 6, 12, and 24 hr (N = 3 at each time period). For multiple dose experiments, DCA was given every 6 hr for three doses and groups of three animals were killed at 3, 6, 12, and 24 hr following each dose. For chronic DCA therapy, animals were dosed daily for 7 days and the rats were killed at 3, 6, 12, 24, 48, and 72 hr after the final DCA dose (N = 3 at each time period). Basal metabolite levels were determined on six animals killed at the start of the experiment. Doses of DCA were staggered such that the time of sacrifice during the day was approximately the same.

The animals were anesthetized with sodium pentobarbital and were exsanguinated via the abdominal aorta. Blood was deproteinized with 4% trichloroacetic acid, neutralized with potassium carbonate, and refrigerated. Whole blood glucose [20] and lactate [21] and pyruvate [22] concentrations were measured enzymatically. Livers were excised, blotted, weighed, freeze-clamped in liquid N_2, and stored at −70° until assayed. One hundred milligrams of frozen liver was used for determination of $PDHC_a$ and $PDHC_t$ activities as described above.

For protein synthesis experiments, animals were divided into control, DCA, inhibitor, and DCA + inhibitor groups. DCA (100 mg·kg^{-1}·dose^{-1}) was administered by gastric intubation at 3, 11, 19, and 27 hr prior to killing the animals. Puromycin (3 mg/kg as a single dose 1 hr prior to the initial DCA dose) or actinomycin D (0.083 mg/kg 1 hr prior to each DCA dose) was injected intraperitoneally.

Statistical analysis. Statistical significance between treated and control groups was determined by Student's *t*-test.

RESULTS

In vivo *studies*

A single dose of DCA caused no significant increase in liver PDHC activation at any sampling time (Fig. 1). At 3 hr there was a significant lowering of blood glucose, lactate and pyruvate ($P < 0.05$) with return to basal levels at 6 hr.

Multiple dosing of DCA every 6 hr caused a progressive rise in PDHC activation with each dose with return to basal levels 24 hr after the second and third doses (Fig. 2).

Figure 3 shows the effect of DCA (100 mg/kg), administered intragastrically for 7 days to fed rats, on blood glucose, blood lactate, and blood pyruvate concentrations. Glucose concentrations were below the basal level ($P < 0.05$) 3 hr after the final DCA dose but returned to basal within 6 hr. Blood lactate was significantly ($P < 0.02$ to < 0.01) below control levels throughout the first 24 hr following drug administration. Blood pyruvate levels were reduced ($P < 0.05$ to < 0.01) below basal for the first 6 hr after the last DCA dose and rose to a level significantly ($P < 0.02$) greater than control values 3 days following cessation of treatment.

Table 1 summarizes the effects of chronic DCA administration on hepatic $PDHC_a$ and $PDHC_t$ activities. Livers were excised 3 hr after the last DCA dose, at a time when the effects of the drug on blood glucose, lactate, and pyruvate were most pronounced

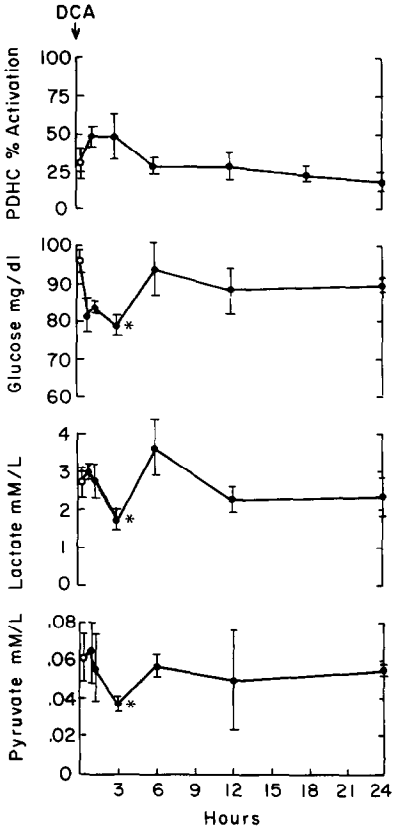

Fig. 1. Effect of a single DCA dose (100 mg/kg) on liver PDHC activation and blood glucose, lactate and pyruvate concentrations. PDHC activation was calculated as $PDHC_a/PDHC_t \times 100$. Each value is the mean ± S.E.M. for three animals. Key: (○) basal; (●) experimental; and (*) $P < 0.05$.

(Fig. 1). Control liver $PDHC_a$ activity averaged 0.70 ± 0.06 nmoles·min^{-1}·(mg protein)$^{-1}$, representing approximately 30% of $PDHC_t$ activity. DCA increased $PDHC_a$ 3.8-fold ($P < 0.001$), and to over 90% ($P < 0.01$) of total enzyme activity. DCA also increased ($P < 0.05$) $PDHC_t$ 26% above control levels.

The duration of the effects of chronic DCA treatment on liver PDHC activity is illustrated in Fig. 4. DCA maintained $PDHC_a$ activity at a level 4-fold greater than control for at least 6 hr after drug administration before enzyme activity returned to control levels. Total PDHC activity showed a significant rise in DCA-treated animals by 3 hr ($P <$

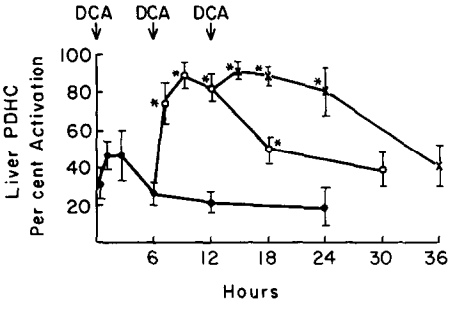

Fig. 2. Effect of multiple DCA dosing (100 mg·kg^{-1}·dose^{-1}) on rat liver PDHC percent activation. PDHC activation was calculated as $PDHC_a/PDHC_t \times 100$. Each value is the mean ± S.E.M. for three animals. Key: (●) single dose; (○) two doses; (×) three doses; and (*) $P < 0.05$.

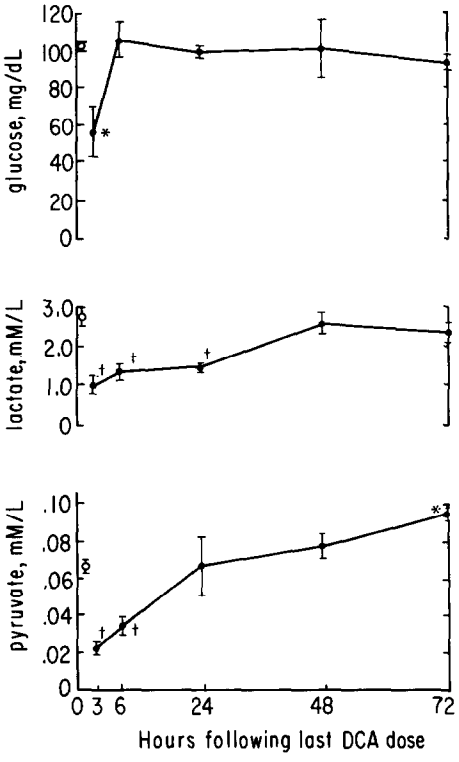

Fig. 3. Effect of DCA on blood glucose, lactate, and pyruvate in healthy, fed rats. DCA was administered intragastrically once daily for 7 days at a dose of 100 mg/kg. Each value is the mean ± S.E.M. for three animals. Key: (○) basal; (●) experimental; (*) $P < 0.05$; and (†) $P < 0.01$.

Table 1. Effect of chronic DCA administration on rat liver PDHC activity*

Group (N)	PDHC [nmoles·min^{-1}·(mg protein)$^{-1}$] $PDHC_a$	$PDHC_t$	% in Active form
Control (5)	0.70 ± 0.060	2.32 ± 0.16	30.7 ± 3.2
DCA (5)	2.71 ± 0.23 ($P < 0.001$)	2.94 ± 0.15 ($P < 0.05$)	91.8 ± 5.5 ($P < 0.01$)

* Rats were treated with DCA, 100 mg·kg^{-1}·day^{-1}, for 7 days as a single dose by gastric intubation. Three hours following the final dose, the animals were killed, and the livers were assayed for PDHC activity as described in the text. The percent in active form was calculated as $PDHC_a/PDHC_t \times 100$. Results are given as mean values ± S.E.M.

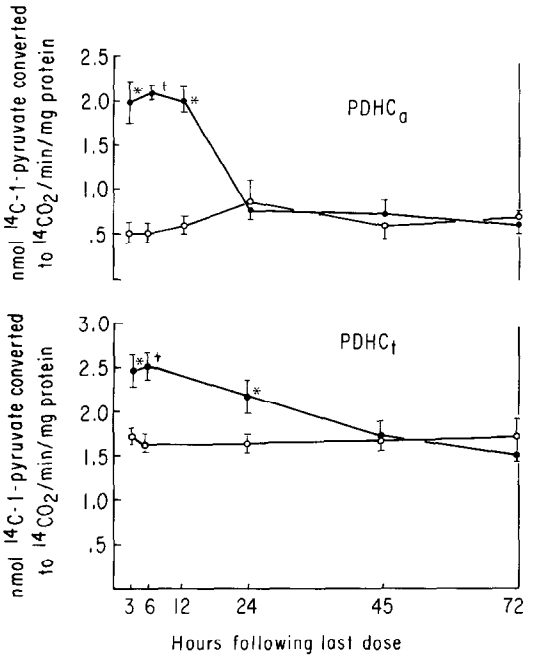

Fig. 4. Effects of chronic DCA administration on liver PDHC activity. Rats were dosed and killed as described in the legend of Fig. 1 and the text. Key: (○) control; and (●) treated. Each value is the mean ± S.E.M. for three animals. P values are < 0.05 (*), and < 0.01 (†).

0.05) which persisted for 24 hr ($P < 0.05$) following the final drug dose. Blood pyruvate concentrations and PDHC$_a$ activity returned to basal levels at 24 hr following chronic DCA administration. Blood lactate concentration returned to basal levels 48 hr following chronic DCA administration at the time when PDHC$_t$ also returned to basal activity (Figs. 3 and 4).

The effect of chronic DCA treatment on the activities of the component enzymes, LAD and PDC, of the PDHC and on the activity of PC, which is inhibited by oxalate [10], is shown in Table 2. DCA did not alter hepatic LAD or PC activity. In contrast, PDC, the rate-limiting enzyme of PDHC [5], showed a 2-fold increase in activity in response to DCA as compared to control values.

To determine if the stimulation of PDHC$_t$ by DCA was due to an effect on enzyme synthesis, rats were treated with puromycin or actinomycin concomitantly with DCA. Preliminary data showed that the increased PDHC$_t$ by DCA could be observed following multiple doses over 24 hr (data not shown). As illustrated in Fig. 5, the stimulatory effect of DCA on PDHC$_t$ was not blocked by either protein synthesis inhibitor. Like DCA, actinomycin increased PDHC$_t$ but, unlike DCA, did not enhance PDHC$_a$ activity.

In vitro *studies*

To determine whether the sustained increase in PDHC$_t$ activity was due to a direct effect of DCA or to its metabolites, glyoxylate and oxalate, on the enzyme, hepatocytes were incubated with various concentrations of DCA, glyoxylate and oxalate. As shown in Table 3, DCA, over the concentration range of 0.5 to 10 mM, stimulated [^{14}C-1]pyruvate oxidation in intact hepatocytes. The effect was dose dependent between 0.5 and 5.0 mM DCA. In contrast, glyoxylate and oxalate inhibited [^{14}C-1]pyruvate oxidation over an 0.1 to 10 mm concen-

Table 2. Effect of chronic DCA administration on rat liver pyruvate decarboxylase (PDC), lipoamide dehydrogenase (LAD), and pyruvate carboxylase (PC)*

Group	N	Enzyme activity [nmoles substrate converted to product · min^{-1} · (mg protein)$^{-1}$] PDC	LAD	PC
Control	3	0.098 ± 0.015	243 ± 12.3	54.8 ± 14.5
DCA	3	0.219 ± 0.013 ($P < 0.01$)	251 ± 10.7	62.4 ± 3.2

* Rats were treated and killed as described in Table 1 and the text. Results are given as mean values ± S.E.M.

Table 3. The effects of DCA, glyoxylate, and oxalate on [^{14}C-1]pyruvate flux through PDHC in isolated rat hepatocytes*

Compound added	[^{14}C-1]pyruvate oxidation (% of control) Drug concentration (mM) 0.01	0.10	0.50	1.0	5.0	10.0
DCA	100.4 ± 9.7	98.1 ± 18.6	137.6 ± 26.2	152.5 ± 22.8	181.1 ± 16.5	150.8 ± 10.8
Glyoxylate	95.5 ± 8.8	64.6 ± 14.6	57.9 ± 8.0	35.6 ± 9.4	26.1 ± 3.2	0
Oxalate	88.6 ± 10.9	70.8 ± 8.3	58.6 ± 6.0	66.7 ± 8.4	72.5 ± 9.1	31.8 ± 6.1

* Hepatocyte suspensions were incubated with or without added drug as described in the text and the $^{14}CO_2$ liberated from [^{14}C-1]pyruvate was used to estimate flux through PDHC. The results are expressed as the mean (drug/control × 100) ± S.E.M. for five experiments. The absolute value for the control mean was 6.32 ± 0.57 nmoles · min^{-1} · (10^{-6} cells)$^{-1}$.

Table 4. Effects of DCA, glyoxylate, and oxalate on hepatocyte PDHC activity*

Compound added	PDHC activity (% of control)					
	Drug concentration (mM)					
	0.01	0.10	0.50	1.0	5.0	10.0
DCA	95.4 ± 11.2	115.2 ± 10.8	123.9 ± 7.4	130.6 ± 6.9	141.2 ± 7.5	159.1 ± 6.8
Glyoxylate	79.0 ± 2.0	61.4 ± 15.6	66.5 ± 23.5	45.3 ± 1.7	55.4 ± 1.7	37.0 ± 6.8
Oxalate	90.7 ± 11.8	81.3 ± 9.9	71.3 ± 9.4	77.9 ± 7.5	57.2 ± 8.6	42.0 ± 5.4

* Hepatocyte suspensions were incubated with or without added drug and PDHC activity was determined as described in the text. Each result is the mean (drug/experimental × 100) ± S.E.M. for five experiments. The absolute value for control mean was 1.73 ± 0.04 nmoles·min^{-1}·(10^6 cells)$^{-1}$.

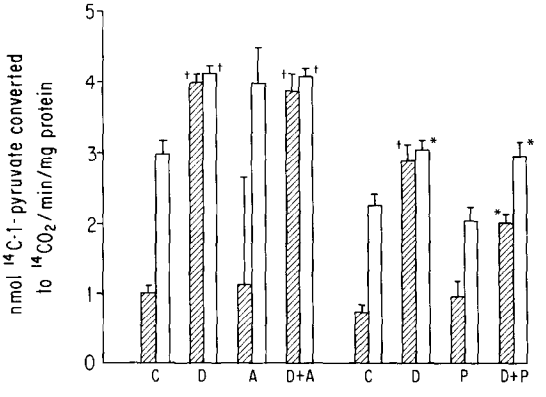

Fig. 5. Effects of protein synthesis inhibitors on DCA-induced increased liver PDHC activity. Rats were treated and killed as described in the text. Hatched bars, $PDHC_a$; open bars, $PDHC_t$. Each result is the mean ± S.E.M. for five animals. P values < 0.05 (*); and < 0.01 (†). Key: (C) control; (D) DCA; (A) actinomycin; and (P) puromycin.

tration range. Similarly, as shown in Table 4, DCA produced a dose-dependent increase in $PDHC_a$ activity, whereas glyoxylate and oxalate were inhibitory at these concentrations. The inhibitory effects of oxalate may be secondary to its complexing of Mg^{2+} [23].

DISCUSSION

Since the discovery by Lorini and Ciman [24] of the blood glucose-lowering effects of DCA in experimental diabetes, the drug has been a useful tool in the study of intermediary metabolism. Whitehouse and coworkers showed that DCA stimulated PDHC activity [7] by inhibiting pyruvate dehydrogenase kinase [8]. Activation of PDHC *in vivo* occurs within minutes of a single DCA dose, and the decline in circulating levels of glucose, lactate, and alanine is correlated with the rise in PDHC activity [8]. In addition, DCA stimulated PDHC activation in a number of tissues including epididymal fat pad, perfused heart and diaphragm [8]. Skeletal muscle PDHC activation also parallels liver activity with chronic DCA therapy.* As shown in Table 2, the increase in PDHC activity was due to the stimulation of PDC, the rate-limiting enzyme of the PDHC system [5]. The activities of PDC and PDHC reported here are not comparable, however, because of the differences in the assay techniques.

Acute or chronic DCA administration reduces blood glucose levels in diabetic [24] or starved [25] animals. In addition, our results show that chronic DCA administration to healthy, fed rats transiently but significantly lowers blood glucose concentrations (Fig. 1). Similar findings in normal dogs have been reported recently by Ribes *et al.* [2]. Several mechanisms may account for the hypoglycemic effect of DCA in healthy, fed animals, such as stimulation of glucose clearance and glycolysis by peripheral tissues [1, 26, 27], reduction of gluconeogenesis by limiting the provision from peripheral sites of 3-carbon gluconeogenic precursors [9, 25, 28], and by direct or indirect inhibition of glucose synthesis at the hepatocyte level [10, 13, 28].

Our study also shows that chronic DCA treatment significantly increased hepatic $PDHC_a$ and $PDHC_t$ activities in fed rats (Table 1 and Fig. 4). The time course of stimulation of $PDHC_t$ activity *in vivo* paralleled closely the onset and duration of the fall in the plasma lactate level. Blood lactate values returned to basal value as $PDHC_t$ activity declined to normal over a 48 hr period. The rise in $PDHC_t$ activity produced by DCA was not blocked by protein synthesis inhibitors (Fig. 5). Likewise, the prolonged stimulatory effect of DCA was not due to the accumulation of active metabolites, since studies with isolated hepatocytes showed that glyoxylate and oxalate inhibited both [^{14}C-1]pyruvate oxidation and $PDHC_a$ activity (Tables 3 and 4). Finally, although DCA inhibited pyruvate dehydrogenase kinase, this effect would serve only to increase the portion of PDHC in an active, dephosphorylated, form and should not alter total enzyme activity, *per se*. We conclude, therefore, that DCA itself directly increased total PDHC activity. The mechanism for this stimulation requires further study.

* Manuscript submitted for publication. These data were presented as an abstract at the 33rd annual meeting of the American Academy of Neurology, 1981. Liver and muscle PDHC activity showed similar activation following chronic DCA therapy, and both tissues had similar tissue DCA concentrations.

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