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Inhibition of Pyruvate Dehydrogenase and Pyruvate Dehydrogenase Phosphate Phosphatase by Glyoxylate[1]

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The overall reaction catalyzed by the pyruvate dehydrogenase complex from rat epididymal fat tissue is inhibited by glyoxylate at concentrations greater than 10 μM. The inhibition is competitive with respect to pyruvate; K_i was found to be 80 μM. Qualitatively similar results were observed using pyruvate dehydrogenase from rat liver, kidney, and heart. Glyoxylate also inhibits the pyruvate dehydrogenase phosphate phosphatase from rat epididymal fat, with the inhibition being readily detectable using 50 μM glyoxylate. These effects of glyoxylate are largely reversed by millimolar concentrations of thiols (especially cysteine) because such compounds form relatively stable adducts with glyoxylate. Presumably these inhibitions by low levels of glyoxylate had not been previously observed, because others have used high concentrations of thiols in pyruvate dehydrogenase assays. Since the inhibitory effects are seen with suspected physiological concentrations, it seems likely that glyoxylate partially controls the activity of pyruvate dehydrogenase *in vivo*.

Generally, glyoxylate acts as an inhibitor of oxidative metabolism. It is toxic to animals (*1*), and it has been shown to inhibit respiration in numerous systems *in vitro* (*2–6*). Glyoxylate could be exerting its effects at several levels. In concert with oxaloacetate, it inhibits aconitase (*7, 8*), $NADP^+$-specific isocitrate dehydrogenase (*9, 10*), and α-ketoglutarate dehydrogenase (*9*). Also, glyoxylate inhibits mitochondrial phosphate transfer, as well as mitochondrial substrate and/or electron transport (*5*). The glyoxylate inhibition of certain tricarboxylic acid cycle enzymes is suspected to contribute to cardiac failure in thiamine-deficient animals (*11*). Glyoxylate builds up in the tissues (*12, 13*), urine, and blood (*12*) of thiamine-deficient rats, and has been detected in the blood of 2 out of 10 patients with thiamine deficiency in which fluid retention and cardiac failure were the predominant symptoms (*14*).

Findings such as the above imply that glyoxylate may be an important effector of various physiological processes. This implication has been strengthened by recent investigations in our laboratory (*15–24*). We have found that adducts of glyoxylate with various nucleophiles are the most likely physiological substrates for the peroxisomal oxidases, D-amino acid oxidase, D-aspartate oxidase, and L-

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hydroxy acid oxidase. Furthermore, we have summarized considerable circumstantial evidence that the products of these reactions, especially of the D-amino acid oxidase reaction (*18, 20, 23*), may participate in the control of metabolism in animals. As a result of these findings, we have become interested in the effects of both glyoxylate and the products of the peroxisomal oxidase reactions on various enzymes, and we report here the effect of glyoxylate on the activity of the pyruvate dehydrogenase complex (PDC)[3] from rat epididymal tissue. Although it had been reported (*25, 26*) that glyoxylate inhibits the overall reaction catalyzed by PDC only at relatively high concentrations, those experiments were carried out with excess thiol present, conditions where most of the glyoxylate would be present as an adduct (*22*). We find that, at low thiol concentrations, glyoxylate is a potent inhibitor of the overall PDC reaction and also of the phosphatase associated with the complex.

EXPERIMENTAL PROCEDURES

Materials. Male Wistar rats (200–250 g) were purchased from Hill Top Lab Animals Inc. (Scottsdale, Pa.). Cysteine hydrochloride and glyoxylic acid monohydrate were purchased from Aldrich. Glyoxylic acid was standardized by the method of Lewis and Weinhouse (*27*). Sodium pyruvate, coenzyme A, thiamine pyrophosphate, nicotinamide adenine dinucleotide, dithiotreitol, β-mercaptoethanol, ethylene glycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic (EGTA) acid, EDTA, magnesium chloride, Triton X-100, β-D-glucose, lyophilized yeast hexokinase, type II collagenase, sucrose, bovine serum albumin, and potassium oxalate were obtained from Sigma. Calcium chloride was purchased from Fisher, and [1-^{14}C]sodium pyruvate from New England Nuclear. Hydroxide of hyamine 10-X was obtained from Packard. All other materials were from standard sources.

Protein determination. Protein concentrations were measured by the method of Lowry *et al.* (*28*), using bovine serum albumin as standard.

Homogenates. Rats were sacrificed by decapitation. The tissues were removed and stored at 4°C until use later the same day. Minced tissues were homogenized in the ratio of 1 g tissue to 4 ml buffer with three strokes of a glass–Teflon homogenizer. The homogenization buffer varied, and will be described for each experiment. The homogenate was then centrifuged at 754*g* for 2 min in the case of fat homogenate, and 10 min for liver, kidney, and heart. The upper fatty layer was removed from the epididymal fat homogenate, and the infranatant was used in further experiments. The supernatants from the homogenates of liver, kidney, and fat were used in further experiments.

Isolated fat cell mitochondria. Preparation of an isolated fat cell mitochondrial fraction was carried out according to the procedure of Jarett (*29*). The unwashed mitochondrial fraction was stored at −20°C in 30 mM potassium phosphate buffer, pH 7.0, containing 1 mM β-mercaptoethanol, and was used without further purification.

[3] Abbreviations used: PDC, pyruvate dehydrogenase complex; kat, katal; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)*N,N'*-tetraacetic acid.

Assays. Pyruvate dehydrogenase was assayed at 37°C by measuring the release of $^{14}CO_2$ using [1-^{14}C]pyruvate as substrate. Unless otherwise specified, each assay contained 1 mM TPP, 0.5 mM NAD^+, 0.1 mM CoA, 1.5 mM $MgCl_2$, 35–50 μM [1-^{14}C]pyruvate (7.9 mCi/mol), and 50 mM potassium phosphate, pH 7.0, in a total volume of 0.5 ml. All the assay components except protein samples were added to 25-ml reaction flasks and kept on ice. Before the assays, the reaction flasks and assay components were warmed for 1 min at 37°C in a constant-temperature shaker bath. Reactions were initiated by addition of protein solution and, in blank reactions, protein was replaced with the buffer in which the protein solution was prepared. The flasks were immediately fitted with top stoppers and center wells, containing 40 μl hydroxide of hyamine. After 2 min the reactions were quenched by injection of 0.6 ml 6 N H_2SO_4 through the top stoppers. Shaking at 37°C was continued at least 90 min. Finally, the center wells were removed and the radioactivity was measured by liquid scintillation counting. Blanks gave less than 20% of the $^{14}CO_2$ yield observed in enzymic reactions. Specific activity is given in katals/kg; 1 katal (kat) is equal to 1 mol/sec.

A modification of the procedure of Mukherjee and Jungas (*30*) was used to measure pyruvate dehydrogenase phosphate phosphatase activity. This assay is based on the rate of activation of pyruvate dehydrogenase in the presence of Mg^{2+}

TABLE 1

GLYOXYLATE INHIBITION OF PDC FROM RAT EPIDIDYMAL FAT TISSUES[a]

[Glyoxylate] (mM)	Percentage inhibition using PDC in		
	Homogenate with mitochondria intact[b]	Homogenate with broken mitochondria[b]	Isolated mitochondria[c]
0.010	14	14	—
0.025	24	26	16
0.050	38	44	32
0.100	53	66	45
0.25	76	83	65
0.50	89	91	84
0.75	92	95	92
1.0	94	96	98
10.0	100	100	—

[a] The assays were carried out as described under Experimental Procedures, with 40 μM pyruvate present. In the assays involving the crude homogenates 0.05 mM DTT was present, and in those using isolated mitochondria 0.1 mM mercaptoethanol was present.

[b] The homogenates were prepared using 10 mM potassium phosphate buffer, pH 7.0, 0.1 mM DTT, 1 mM EDTA, and either 0.25 M sucrose (to keep the mitochondria intact) or 0.05% Triton X-100 (to break the mitochondria). The specific activities of these preparations were 15 and 12 μkat/kg, respectively, without any glyoxylate present.

[c] The isolated mitochondria had a specific activity of 108 μkat/kg without glyoxylate present.

and Ca^{2+}. The homogenate was incubated at 30°C in the presence of 10 mM D-glucose, 1 unit (1 μmol/min) hexokinase/ml, 1.5 mM $MgCl_2$, and 1.75 mM $CaCl_2$. All incubations contained 0.5 mM DTT and 1 mM EGTA (present in the homogenization buffer). At the specified time intervals, portions were removed and assayed for pyruvate dehydrogenase activity.

RESULTS

Glyoxylate inhibition of the overall PDC reaction. In homogenates prepared from various rat tissues it was found that glyoxylate at concentration of 10 μM or more causes measurable inhibition of the PDC activity. Given in Table 1 are some results obtained using PDC from rat epididymal fat tissue. These assays were performed under conditions where the amounts of PDC in the phosphorylated and dephosphorylated states would not be appreciably altered during the assay, so the effects seen are due to effects of glyoxylate on the rate of the actual conversion of pyruvate to acetyl-CoA and CO_2 catalyzed by PDC. As can be seen from the results in Table 1, quantitatively similar inhibition was obtained using intact or broken mitochondria in crude homogenates or using isolated mitochondria. The percentage inhibition in the latter is slightly less, probably because a higher concentration of thiol (0.1 mM rather than 0.05 mM) is present in the assay. The

TABLE 2

GLYOXYLATE INHIBITION OF PDC FROM RAT HEART, LIVER, AND KIDNEY[a]

[Glyoxylate] (mM)	Percentage inhibition using homogenate from		
	Heart	Liver	Kidney
0.05	32	0	8
0.10	40	13	13
0.25	46	47	51
0.50	58	66	78
0.75	66	86	79
1.0	84	85	86
2.5	—	—	97
5.0	100	100	99
10	100	100	100

[a] Assayed as described under Experimental Procedures, with 65 μM pyruvate present. The concentration of DTT in the assays of the heart and liver homogenates was 0.05 mM, and it was 0.016 mM in the assay of the kidney homogenate. The homogenates were prepared using 10 mM potassium phosphate buffer, pH 7.0, 0.1 mM DTT, and 1 mM EDTA. The specific activities of the homogenates without glyoxylate present were heart, 2; liver, 0.23; and kidney, 11 μkat/kg.

TABLE 3

EFFECTS OF COFACTOR CONCENTRATIONS ON THE INHIBITION OF PDC BY GLYOXYLATE[a]

[NAD^+] (μM)	[CoA] (μM)	[TPP] (μM)	Specific activity with no glyoxylate (μkat/kg)	Percentage inhibition with	
				50 μM glyoxylate	100 μM glyoxylate
500	100	1000	100	29	46
50	100	1000	86	30	46
10	100	1000	56	25	42
500	20	1000	81	36	51
500	5	1000	53	38	52
500	100	100	103	35	48
500	100	10	94	37	55
500	100	1	53	36	59
500	100	0.1	17	49	68

[a] Assayed as described under Experimental Procedure, using isolated mitochondria from rat epididymal fat and with 40 μM pyruvate, 0.1 mM 2-mercaptoethanol, and the cofactors at the given concentrations.

PDCs in rat heart, liver, and kidney homogenates are also inhibited by glyoxylate (Table 2) but they do not appear to be quite as sensitive as the PDC from epididymal fat.

The glyoxylate inhibition of PDC from epididymal fat is not very dependent on the concentrations of the cofactors employed. As illustrated by the results in Table 3, these can be changed by more than an order of magnitude with very little effect on the percentage inhibition caused by a given concentration of gly-

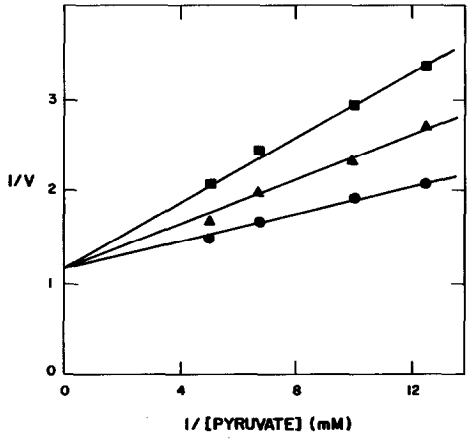

FIG. 1. Effect of pyruvate concentration on the glyoxylate inhibition of PDC from rat epididymal fat. The experiments were carried out using homogenates prepared using 10 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA, 0.1 mM DTT. PDC activity was assayed as described under Experimental Procedures; ●, no inhibitor; ▲, 50 μM glyoxylate; ■, 100 μM, glyoxylate.

oxylate when a particular concentration of pyruvate is present. However, the amount of inhibition is dependent on the pyruvate concentration, and as the results in Fig. 1 illustrate, the glyoxylate inhibition is competitive with respect to pyruvate. From these data the K_m for pyruvate is calculated to be 63 μM and the K_i for glyoxylate, 80 μM.

Glyoxylate reversibly forms covalent thiohemiacetal adducts with thiols (*22*), and to the extent that this occurs it will effectively lower the concentration of glyoxylate in solution. Thus, it is not surprising that the inhibition of PDC by glyoxylate is largely reversed (Table 4) as the concentration of thiol in the assay medium is increased. The presence of some thiol during homogenization is necessary to keep PDC active, and CoA must be present during the assay, but it is obvious from Table 4 that the presence of excessive amounts of thiols during PDC assays will effectively mask the glyoxylate inhibition.

Inhibition of PDC phosphatase by glyoxylate. The PDC in homogenates of epididymal fat from starved rats was largely in the phosphorylated, catalytically inactive form. As shown by the results in Fig. 2, glyoxylate slows the rate of conversion of this form to the catalytically active species when the homogenates are incubated with Mg^{2+} and Ca^{2+}, metal ions that activate the PDC phosphatase. These results indicate that glyoxylate is inhibiting the PDC phosphatase. That the effect of glyoxylate is an effect on the phosphatase and not only on the overall PDC reaction is evident from (a) the difference between the PDC activity with and without glyoxylate present during incubation is greater following activation by Mg^{2+} and Ca^{2+}, and (b) if additional Mg^{2+} and Ca^{2+}, as well as 5 mM cysteine (to effectively reverse the glyoxylate effect), are added to the homogenate undergoing activation, the homogenate with glyoxylate reaches a final PDC activity that corresponds to fully activated PDC. Lower PDC activity is still observed in the presence of glyoxylate, but is no lower than would be expected for direct inhibition of PDC under the assay conditions.

TABLE 4

ATTENUATION OF GLYOXYLATE INHIBITION OF PDC FROM RAT EPIDIDYMAL FAT BY THIOLS[a]

[DTT] (mM)	[Cysteine] (mM)	Percentage inhibition with 50 μM glyoxylate	Percentage inhibition with 500 μM glyoxylate
0.05	0	36	85
1.0	0	18	—
0.05	5.0	10	47
0.05	10.0	—	38

[a] Homogenates prepared using 30 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA, 50 mM NaCl, and 0.1 to 0.5 mM DTT were assayed as described under Experimental Procedures, using 40 μM pyruvate. The specific activity of the homogenates without glyoxylate present was 9 to 12 μkat/kg.

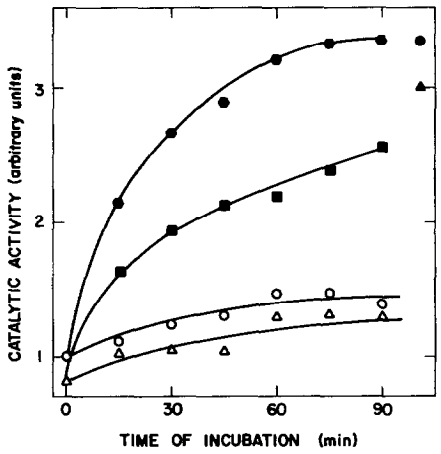

FIG. 2. The effect of glyoxylate on PDC phosphatase in rat epididymal fat homogenates. Prior to sacrifice rats were deprived of food for 24 hr. The homogenates were prepared in 10 μM potassium phosphate buffer, pH 7.0, 1 mM EGTA, 0.5 mM DTT. Following incubation at 30°C for the indicated length of time under the conditions given under Experimental Procedures, aliquots were removed and assayed for PDC activity with 5 mM cysteine present (to minimize glyoxylate inhibition of the overall PDC reaction so that the inhibition of the phosphatase could be more readily detected). Incubation conditions: ●, 1.5 mM Mg^{2+}, 1.75 mM Ca^{2+}; ■, 1.5 mM Mg^{2+}, 1.75 mM Ca^{2+}, 100 μM glyoxylate; ○, no Mg^{2+} or Ca^{2+}; △, no Mg^{2+} or Ca^{2+}, 100 μM glyoxylate; ●, 1.5 mM Mg^{2+}, 1.75 mM Ca^{2+} for 60 min, and 8.0 mM Mg^{2+}, 2.25 mM Ca^{2+}, 5 mM cysteine for the rest of the time; ▲, 1.5 mM Mg^{2+}, 1.75 mM Ca^{2+}, 100 μM glyoxylate for 60 min, and 8.0 mM Mg^{2+}, 2.25 mM Ca^{2+}, 100 μM glyoxylate, 5 mM cysteine for the rest of the time.

Data collected from six experiments identical to the one given in Fig. 2 are summarized in Table 5. These results emphasize that the difference in PDC activity with and without glyoxylate (indicated as percentage apparent inhibition of PDC) is greater following activation, and thus that glyoxylate is inhibiting

TABLE 5

THE EFFECT OF GLYOXYLATE ON PDC PHOSPHATASE IN RAT EPIDIDYMAL FAT HOMOGENATES

Length of incubation (min)	Percentage apparent inhibition of PDC by glyoxylate[a]	
	no Mg^{2+} or Ca^{2+}	with Mg^{2+} and Ca^{2+}
0	10 ± 6	—
15	10 ± 4	25 ± 4
30	8 ± 5	22 ± 4
45	12 ± 5	24 ± 2
60	11 ± 5	26 ± 3

[a] The glyoxylate concentration during the incubation was 100 μM, and during the PDC assay μM. The data presented are averages (± the standard deviation) of six identical experiments performed as described in Fig. 2.

the phosphatase. In other experiments (data not shown) it was found (*31*) that inhibition of the phosphatase is readily detectable with 50 μM glyoxylate present during activation, but with 25 μM glyoxylate the differences are not significant. Also, the effect is not dependent on Ca^{2+}, because similar inhibition of the phosphatase is observed when it is activated by 4 mM Mg^{2+} alone (*31*). Finally, if the homogenate is preincubated for 30 min with 100 μM glyoxylate in the absence of metal ion activators, and then 10 mM cysteine is added along with Mg^{2+} and Ca^{2+}, very little inhibition of the phosphatase is detectable following subsequent incubation (*31*). This emphasizes that glyoxylate has no irreversible effect on the phosphatase; if it is removed by combination with cysteine then virtually normal activation ensues.

DISCUSSION

The present study has shown glyoxylate to be a good inhibitor of the overall reaction catalyzed by rat epididymal fat PDC; it is competitive with pyruvate and gives a K_i of 80 μM at pH 7.0. Although PDCs from rat heart, liver, and kidney do not appear to be quite as sensitive to glyoxylate, they are still inhibited approximately 50% by 250 μM glyoxylate when pyruvate is present at a concentration approximately equal to its K_m. The detailed mechanism of the inhibition has not been studied in this investigation, but since it does not depend to any great extent on the concentrations of the cofactors, one suspects that glyoxylate is just competing with pyruvate for binding to the complex and possibly by acting as a poor substrate.

Strong inhibition of yeast PDC by glyoxylate was noted as early as 1943 by Kleinzeller (*3*). However, it was subsequently reported that glyoxylate only inhibits at relatively high concentrations. Thus, Walsh *et al.* (*25*) reported that the K_i for pig heart PDC was 0.56 mM, and Rubin *et al.* (*26*) reported a glyoxylate K_i of 3.3 mM for plant PDC. However, both these groups of investigators assayed for the inhibition in the presence of excess thiol, 5 mM 2-mercaptoethanol, and 2.5 mM cysteine, respectively. As reported here, thiols effectively reverse the glyoxylate inhibition, presumably because they form stable hemithioacetal adducts with glyoxylate (*22*). Cysteine forms an especially stable thiazolidine adduct (*24, 32*), so it is not surprising that glyoxylate inhibition was not seen in the Rubin study (*26*) until its concentration was greater than that of the cysteine present. It seems possible that glyoxylate effects on other enzymes may also have been missed because they were assayed with high concentrations of thiols present.

The inhibition of PDC phosphatase by glyoxylate, as found here, had not been reported previously. Since inhibition is detectable at 50 μM glyoxylate, it represents a fairly potent inhibition. Because glyoxylate has very little effect on the activity of PDC kinase (*33*), this finding therefore indicates that the level of glyoxylate will partially determine the ratio of active to inactive forms of PDC; high glyoxylate concentrations will lead to more of the inactive phosphorylated form. It seems likely, though not proven by the present results, that glyoxylate may be modifying the activity of the phosphatase by forming a thiohemiacetal

with a reactive enzymatic thiol group; there is evidence (*4*) that the enzyme has such a group.

Since the concentration of glyoxylate in cells is thought to be normally in the range 10–100 μM, and even higher in thiamine deficiency (*12*), it is likely from the present results that the activity of PDC and its phosphatase will be partially controlled physiologically by the level of glyoxylate. Therefore, more attention should probably be focused on glyoxylate as a possible *in vivo* PDC effector, including possibly participating as part of the intracellular response to insulin. Insulin is known to activate PDC apparently by increasing the activity of the phosphatase (*35*), but the details by which it might do this by causing a decrease in the intracellular glyoxylate concentration remains to be elucidated. Another mechanism by which insulin could affect this enzyme has recently been articulated (*23*).

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