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INHIBITION OF PYRUVATE CARBOXYLASE BY CHLOROPYRUVIC ACID AND RELATED COMPOUNDS

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Abstract—3-Chloro-1,2-propanediol (CPD), a compound with male antifertility activity in several species, was slowly metabolized in rat liver slices. Incorporation of $^{14}CO_2$ into glucose in slices incubated with lactate or pyruvate was inhibited by CPD and by chloropyruvic acid, a possible metabolite. At 0·1 mM, chloropyruvic acid (CPA) and related compounds, including beta-chlorolactic, fluoropyruvic and chloroacetic acid, inhibited incorporation into glucose of label from [U-^{14}C]alanine without affecting production of ketone bodies or $^{14}CO_2$. Inhibition was not observed in similar preparations using succinate as substrate. In mitochondria, concentrations of 1–10 mM CPA inhibited CO_2 fixation by 56–95 per cent. Relative inhibition was CPA > fluoropyruvic acid > beta-chlorolactic acid > chloroacetic acid. In acetone powder extracts, CPA at 1–10 mM reduced CO_2 fixation by 21–86 per cent. Relative inhibition was CPA > beta-chlorolactic acid > chloroacetic acid. Interconversion between CPA and chlorolactate was not catalyzed by lactic dehydrogenase, nor did these compounds inhibit this enzyme. The data indicate that CPA and related acids may inhibit gluconeogenesis by specific inhibition of pyruvate carboxylase. Whether or not this metabolic effect is involved in the development of sterility after administration of CPD remains to be determined.

DURING a study of the metabolism of 3-chloro-1,2-propanediol (alpha-chlorohydrin; U-5897; CPD), an effective male antifertility agent in the rat, guinea pig and monkey,[1,2] it was noted that high doses markedly inhibit glucose production in rat liver slices. Oxidation of the diol to chloropyruvic, beta-chlorolactic or chloroacetic acid by the slices seemed likely. Therefore these compounds were also tested as potential inhibitors of gluconeogenesis.

Incorporation of ^{14}C from $^{14}CO_2$ into glucose has been shown to reflect hepatic glucose production and is increased in experimental diabetes[3] and by glucocorticoids.[4] Pyruvate carboxylase (EC 6.4.1.1) catalyzes the formation of oxaloacetate from pyruvate and CO_2. Competition with this enzyme by CPD or by the above derivatives, which more closely resemble pyruvic acid, was investigated as a possible mechanism of inhibition of CO_2 fixation.

METHODS

Chemicals other than common reagents were obtained from the following sources: chloropyruvic and chloroacetic acid, Pfaltz & Bauer; beta-chlorolactic acid, K & K Laboratories; sodium pyruvate, Mann Research Laboratories; 3-chloro-1,2-propanediol and fluoropyruvic acid, Aldrich Chemical Company. The first three compounds were purified by vacuum sublimation before use.

Male, Sprague–Dawley rats weighing 200–350 g were used. Animals in liver slice experiments were fasted overnight; others were fed *ad lib.* Rats were sacrificed by stunning and exsanguination.

Liver slices were prepared with a Stadie–Riggs hand microtome, from livers chilled on an ice-cooled petri dish. Approximately 0·5 g of wet tissue was incubated in 5 ml of Krebs–Ringer bicarbonate medium equilibrated with 95% O_2–5% CO_2.

Slices were incubated with 1–20 mM CPD or 0·1 mM CPA or related acid in medium containing 1 mg/ml of substrate. The reaction was stopped by injection of 0·2 ml of 70% $HClO_4$. $^{14}CO_2$ collected in 0·2 ml Hyamine was counted in 10 ml of Bray's solution in a liquid scintillation spectrometer. When $^{14}CO_2$ was not collected, protein was precipitated by heat denaturation followed by precipitation with $ZnSO_4$ and $Ba(OH)_2$.[5] Incorporation of label into glucose was estimated by formation of glucose phenylosazone, which was recrystallized and counted with a Nuclear-Chicago proportional flow counter.[6] Glucose was determined by the glucose oxidase method; ketone bodies (beta-hydroxybutyrate and acetoacetate) were determined by the method of Young and Renold.[7]

For preparation of mitochondria, livers were removed and chilled in 0·3 M sucrose at 0°. Homogenization was carried out in a solution of 0·3 M sucrose–5 mM tris–1 mM EDTA, pH 7·6, using 6 ml buffer per gram of liver, in a motor-driven Potter–Elvehjem type homogenizer with a Teflon pestle. Four passes of the pestle at low revolutions per minute were used. The homogenate was centrifuged at 650 *g* for 10 min. The supernatant was recentrifuged at 10,000 *g* for 20 min. Mitochondrial pellets were combined and resuspended in one-sixth the original volume of buffer, giving a protein concentration of 20–30 mg/ml.

Mitochondrial CO_2 fixation was determined using a mixture of 1 ml of the mitochondrial suspension and 1 ml of sucrose–tris–EDTA buffer enriched with (final concentration): sodium pyruvate, 5 mM; K_2SO_4, 2·5 mM; $KH^{14}CO_3$, 12 mM (1 μc); KH_2PO_4, 8 mM. The compound to be tested was added in 0·1 ml of aqueous solution, with an equivalent of $NaHCO_3$, to give a final concentration of 1–10 mM. The mixture was incubated aerobically at 37° with slow shaking, for 10 min. The reaction was arrested by addition of 4 ml of 5% trichloroacetic acid. Precipitated protein was removed by centrifugation and the solution was gassed with 100% CO_2 for 2 min to remove excess $^{14}CO_2$. An aliquot of the gassed solution was counted in Bray's solution in a liquid scintillation spectrometer.

Hepatic acetone powder extracts were prepared according to the method of Wimhurst and Manchester.[8] The incubation medium for determination of pyruvate carboxylase activity was a modification of that used by the above authors. An aliquot of acetone powder suspension (50 μl) was incubated with 0·65 ml of tris–HNO_3 buffer (0·04 M) enriched with: $KH^{14}CO_3$, 6·6 mM (1·0 μc); $MgSO_4$, 6·1 mM; ATP, 1·7 mM; phosphotransacetylase (Boehringer), 6·6 U; citrate synthetase (Boehringer), 1·2 U; coenzyme A, 0·58 mM; acetyl phosphate, 0·81 mM; and sodium pyruvate, 0·74 mM. The compound to be tested was added as described for mitochondria. After 10 min of aerobic incubation with shaking, the reaction was terminated by addition of 1 ml of 5% trichloroacetic acid. The incubation mixture was gassed and counted as described for mitochondria.

Metabolism of chloropyruvic acid and beta-chlorolactic acid was determined by the method of Bucher *et al.*[9] for the assay of pyruvate, using a Beckman DB spectro-

photometer. In the estimation of metabolism of chloropropanediol by liver slices, diols were measured by the method of Korn.[10] Glucose was measured by the glucose oxidase technique.[11] Protein was measured by the method of Lowry *et al.*[12]

RESULTS

CPD was slowly metabolized in liver slices from fasted rats (Table 1). Disappearance was most rapid during the initial 30 min; relatively little change occurred during the 60 to 90-min interval. Approximately one-fourth of the drug was subject to degradation in the slice preparation.

TABLE 1. METABOLISM OF CHLOROPROPANEDIOL IN LIVER SLICES*

Time (min)	Chloropropanediol (μmoles/ml)	% Metabolized
0	4·53 ± 0·46	0
30	3·89 ± 0·28	13
60	3·38 ± 0·26	24
90	3·28 ± 0·32	27

* Values are expressed as mean ± S.E.; $N = 4$. Liver slices were incubated with 10 mM chloropropanediol. The amount of chloropropanediol was obtained by subtracting the amount of glucose from the total amount of diol in medium.

Inhibition by CPD of CO_2 fixation in liver slices incubated with 10 mM L-lactate was significant at 1–20 mM, and net glucose production was significantly reduced by the presence of 5–20 mM CPD (Table 2). Chloropyruvic acid appears to be more potent than CPD in inhibiting glucose production (Table 3). Marked reduction in CO_2 incorporation into glucose was observed with concentrations of the inhibitor of 0·1–0·5 mM. Similar results were obtained in slices incubated with pyruvate (Table 3).

Chloropyruvic acid and several analogs were also incubated with liver slices in the presence of labeled alanine (Table 4). Addition of pyruvate caused no change in incorporation of label into glucose, oxidation to $^{14}CO_2$, or generation of ketone

TABLE 2. INHIBITION OF CO_2 FIXATION IN LIVER SLICES BY 3-CHLORO-1,2-PROPANEDIOL*

Substrate		Glucose (μmoles/g)	Glucose (counts/min/g)
L-Lactate (10 mM)		68 ± 3·7	61,000 ± 2100
+ Chloropropanediol	(1 mM)	50 ± 8·0	29,000 ± 2300†
	(5 mM)	40 ± 6·8†	14,000 ± 1100†
	(10 mM)	39 ± 8·1†	7200 ± 560†
	(20 mM)	16 ± 1·1†	3000 ± 850†

* Values are expressed as means ± S.E.; $N = 8$.
† $P < 0·01$; 1 μc $NaH^{14}CO_3$ per flask.

TABLE 3. EFFECT OF CHLOROPYRUVIC ACID ON CO_2 FIXATION IN RAT LIVER SLICES*

Substrate	Glucose (μmoles/g)	Glucose (counts/min/g)
Lactate (10 mM)	52 ± 5·6	23,200 ± 1070
+ 0·1 mM CPA	42 ± 5·0	13,100 ± 1300†
+ 0·5 mM CPA	33 ± 4·8	415 ± 24†
Pyruvate (5 mM)	51 ± 5·3	33,450 ± 2900
+ 0·1 mM CPA	35 ± 4·0	15,325 ± 2167†
+ 0·5 mM CPA	28 ± 2·6†	850 ± 183†

* Values are expressed as means ± S.E.; $N = 4$. CPA = chloropyruvic acid.
† $P < 0{\cdot}01$; 1 μc $NaH^{14}CO_3$ per flask.

bodies. Chloropyruvate, beta-chlorolactate and chloroacetate, in contrast, all caused significant inhibition of ^{14}C incorporation into glucose at 0·1 mM. Oxidation of alanine and ketone body production were generally unchanged.

When, in similar experiments, [1,4-$^{14}C_2$]succinate or [2,3-$^{14}C_2$]succinate was substrate, chloropyruvic acid had no effect on incorporation of label into glucose.

Pyruvate carboxylase in rat liver is believed to be a mitochondrial enzyme.[13] It was decided therefore to determine whether the compounds would inhibit CO_2 fixation in suspensions of intact mitochondria (Table 5). The concentration of inhibitor required to inhibit CO_2 fixation in this system was 2–100 times that used in liver slices. Fluoropyruvic acid was roughly comparable to beta-chlorolactic acid in activity. Beta-chloropropanoic acid, in contrast, had no effect.

TABLE 5. INHIBITION OF MITOCHONDRIAL CO_2 FIXATION*

Compound	Per cent inhibition		
	Concentration (mM)		
	1	3	10
Chloropyruvic acid	56·1 ± 7·26	92·3 ± 0·66	96·4 ± 0·74
Fluoropyruvic acid	37·5 ± 7·28	40·7 ± 3·39	83·1 ± 1·92
Beta-chlorolactic acid	45·3 ± 1·42	52·9 ± 2·04	59·5 ± 3·04
Chloroacetic acid	32·3 ± 8·31		53·9 ± 2·37
Beta-chloropropionic acid ($N = 2$)	None	None	None

* Values are expressed as means ± S.E.; $N = 4$, unless otherwise indicated.

Mitochondrial CO_2 fixation may reflect pyruvate carboxylase activity. However, when pyruvate was omitted, fixation never was reduced by more than 30 per cent of control. Endogenous pyruvate is thought to be present at a concentration of up to 0·3 μmoles/g of liver,[14] and to diffuse freely through the mitochondrial membrane.[15] Inasmuch as the reported K_m of pyruvate carboxylase with respect to pyruvate is about 0·3 mM,[8] it is possible that other carboxylation reactions were contributing to CO_2 fixation in the absence of added pyruvate.

TABLE 4. EFFECT OF CHLOROPYRUVIC ACID AND ANALOGS ON INCORPORATION OF ^{14}C FROM [U-^{14}C]L-ALININE INTO GLUCOSE AND CO_2 AND ON PRODUCTION OF KETONE BODIES IN LIVER SLICES*

Substrate	N	Glucose		$^{14}CO_2$		Ketone bodies	
		μmoles/g	counts/min/g	% ^{14}C incorporated	counts/min/g	% ^{14}C incorporated	Aceto-acetate	β-OH-butyrate
Alanine	12	22·2 ± 1·74	12,458 ± 365	9·21 ± 0·486	55,030 ± 1192	12·5 ± 1·10	0·790 ± 0·169	3·46 ± 0·274
+ 0·1 mM Pyruvate	12	24·6 ± 2·74	11,391 ± 404	8·74 ± 0·394	53,980 ± 1904	12·8 ± 1·32	1·23 ± 0·234	3·13 ± 0·291
+ 0·1 mM Chloropyruvate	8	26·8 ± 2·68	6982 ± 490†	5·75 ± 0·482†	47,376 ± 2768	10·0 ± 1·08	0·786 ± 0·042	3·29 ± 0·491
+ 0·1 mM Chlorolactate	6	28·2 ± 3·36	6751 ± 212†	4·87 ± 0·143†	46,580 ± 3127	12·7 ± 2·23	0·863 ± 0·358	5·87 ± 0·726†
+ 0·1 mM Chloroacetate	6	37·4 ± 2·27	9684 ± 424†	7·80 ± 0·570†	54,996 ± 1604	17·1 ± 1·26	1·01 ± 0·344	3·88 ± 0·77

* Values are expressed as means ± S.E.
† $P < 0·01$; 0·2 μc [U-^{14}C]L-alanine per flask.

In acetone powder preparations, at similar concentrations, inhibition of CO_2 fixation was again apparent (Table 6). Thus, the inhibition does not depend on an intact mitochondrial membrane. In the absence of pyruvate, fixation was always less than 10 per cent of control.

TABLE 6. INHIBITION OF CO_2 FIXATION IN ACETONE POWDER EXTRACTS*

Compound	Per cent inhibition		
	Concentration (mM)		
	1	3	10
Chloropyruvic acid	21·4 ± 5·27	61·9 ± 2·33	86·2 ± 3·82
Beta-chlorolactic acid	37·0 ± 8·40	63·8 ± 3·24	85·4 ± 0·79
Chloroacetic acid	10·5 ± 3·88		4·53 ± 9·05

* Values are expressed as means ± S.E.; $N = 4$.

In all of the above experiments, there was the possibility of interconversion of chloropyruvic and beta-chlorolactic acid by lactate dehydrogenase. This was ruled out by the observation that virtually no reaction occurred when either of these substrates was incubated with the enzyme under optimum conditions.[9,16] In addition, neither compound inhibited the interconversion of lactic and pyruvic acid. Therefore it is concluded that neither compound served as a substrate or inhibitor of lactic dehydrogenase.

DISCUSSION

The study of metabolism of CPD in liver slices was limited to a determination of disappearance of CPD. Attempts to use gas chromatography to identify the metabolites formed were frustrated by the thermal instability of the compounds. Metabolism *in vivo* of CPD has been investigated in the rat, rabbit and monkey using (2-^{14}C)CPD.[17] Elimination of ^{14}C after orally administered CPD was estimated in urine (16–26 per cent), faeces (1–3 per cent) and breath (25–32 per cent, as $^{14}CO_2$). Ninety per cent of the radioactivity in the urine represented unchanged drug. Thus, most of the compound was either excreted unchanged or extensively degraded. Assuming that molecular rearrangements did not occur, only a small fraction of the dose could have been converted to metabolites of interest here. Nevertheless, CPD may owe its activity to a potent metabolite present at low concentration. The degree to which metabolism *in vivo* is related to that in the liver slice system is unknown. It is conceivable that in liver slices some of the CPD was oxidized to beta-chlorolactic acid by a nonspecific alcohol dehydrogenase. Conversion of beta-chlorolactic acid to chloropyruvic acid by lactic dehydrogenase seems unlikely.

Chloropyruvic acid was a more potent inhibitor of CO_2 fixation in liver slices incubated with lactate or pyruvate than was CPD, but this may not be relevant to the activity of CPD. At present, there is no evidence of CO_2 incorporation into glucose in the epididymus or of any effect of the compounds tested here on glucose metabolism in isolated epididymal slices (unpublished observations). Thus, no connection has been established between the antifertility activity of CPD and the effects of CPD and its possible metabolites on epididymal carbohydrate metabolism.

Inhibition of pyruvate carboxylase by CPD and related compounds may prove to be a useful tool in the study of the regulation of gluconeogenesis. An example of previous use of an inhibitor in this regard is the inhibition by L-tryptophan (thought to act via an active metabolite, quinolinic acid) of phosphoenolpyruvate carboxykinase, which led to the recognition of a crossover point between oxaloacetate and phosphoenolpyruvate.[18] Lack of effect of CPD and analogs on $^{14}CO_2$ or ketone body formation from [U-^{14}C]alanine in liver slices has been noted. That $^{14}CO_2$ formation from alanine was not inhibited suggests that these compounds do not inhibit pyruvic dehydrogenase or subsequent oxidation of acetyl CoA. When in similar experiments the substrate was succinic acid, which can be converted to oxaloacetic acid without pyruvate carboxylase, chloropyruvic acid caused no inhibition of incorporation of label into glucose. Deletion of pyruvate from acetone powder suspensions in which CO_2 fixation could be markedly inhibited by these chemicals almost completely abolished the fixation. In other experiments, the action of lactic dehydrogenase on pyruvate was unaffected by chloropyruvic or beta-chlorolactic acid. The data are compatible with a specific inhibition by these compounds of pyruvate carboxylase.

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