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FURTHER STUDIES ON THE SITE AND MECHANISM OF ACTION OF *S*-(1,2 DICHLOROVINYL)-L-CYSTEINE AND *S*-(1,2 DICHLOROVINYL)-3-MERCAPTOPROPIONIC ACID IN RAT LIVER

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Abstract—DCVC, but not DCVMP, inhibits lipoyl dehydrogenase activity of isolated rat liver mitochondria and glutathione reductase activity of rat liver cytosol. DCVC can be degraded to pyruvic acid and ammonia by the cytosolic fraction of rat liver. Metabolism of DCVC by the cytosolic fraction appears necessary for inhibition of glutathione reductase activity.

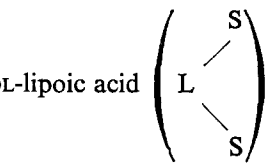
S-(1,2 DICHLOROVINYL)-L-CYSTEINE (DCVC) a compound formed during food processing is nephrotoxic in all mammalian species tested.[1–3] Administration of DCVC to the rat or its addition *in vitro* to mitochondria shows that respiration is impaired.[4] The mitochondrial site of action is the 2-oxoacid dehydrogenases.[5] DCVC produces a delayed response in several *in vitro* systems[5] and this has been correlated with the metabolism of DCVC to an active inhibitor.[6] This view finds further support in this present study.

A compound related to DCVC, namely *S*-(1,2 dichlorovinyl)-3-mercaptopropionic acid (DCVMP) also inhibits mitochondrial respiration *in vivo* and *in vitro*.[4] Although its action is upon the 2-oxoacid dehydrogenase,[5] evidence is presented in this study to show that DCVC and DCVMP do not produce their inhibitory actions at the same sites.

MATERIALS AND METHODS

Isolation of mitochondria. Rat liver mitochondria were prepared from liver in 300 mM sucrose as described by Aldridge.[7]

Preparation and assay of lipoyl dehydrogenase. Rat liver mitochondria were isolated in 150 mM KCl instead of 300 mM sucrose as already described. The suspension was frozen and thawed three times in a flask surrounded by acetone–solid carbon dioxide, sonicated for 1 min in an MSE ultrasonic disintegrator at 1·5 A (60 W) and finally centrifuged at 0° for 30 min at 40,000 *g*. The supernatant was thoroughly drained and the pellet was resuspended in a small volume of ice-cold 150 mM KCl. Preliminary experiments indicated that lipoyl dehydrogenase activity resided entirely in the supernatant fraction.

Lipoyl dehydrogenase activity was assayed spectrophotometrically by following the oxidation of NADH at 340 nm coupled to oxidized DL-lipoic acid  as hydrogen acceptor. Subtraction of the rate of NADH oxidation in the absence of lipoic acid (20 per cent of the total rate) from that in the presence of lipoic acid gives lipoyl dehydrogenase activity. The assay was performed at 25° or 37° on a Unicam SP-800. The assay medium (3 ml total volume) was composed as follows: (Massey[8]); 1 M citrate buffer, pH 5·7; 0·1 mM NADH; 0·1 mM NAD; 0·67 mM-DL-lipoic acid; supernatant fraction.

DCVC and DCVMP were either added immediately to the assay system at 25° and 15–20 μl of supernatant fraction ($\equiv$ 0·23 mg protein) added or were preincubated with a sample of supernatant fraction ($\equiv$ 6–6·5 mg protein) for 30 min at 37° on a metabolic shaker and 15–20 μl of this fraction ($\equiv$ 0·23 mg protein) taken for the assay of lipoyl dehydrogenase activity at 37°.

Preparation and assay of glutathione reductase. Liver (8 g) was homogenized in 30 ml ice-cold 300 mM sucrose. Homogenate was diluted with 35 ml of 300 mM sucrose and was centrifuged in a MSE SS50 for 60 min at 105,000 *g* to give the cytosolic fraction.

Glutathione reductase activity was assayed in the cytosolic fraction of rat liver by measuring the oxidation of NADPH at 340 nm coupled to oxidized glutathione (GSSG) as hydrogen acceptor, under similar conditions to those used by Wendell.[9] No subtraction of the rate of NADPH oxidation in the absence of oxidized glutathione was necessary since the activity was less than 0·5 per cent of that in the presence of oxidized glutathione. The assay was performed at 37° on a Unicam SP-800 in a medium containing 67 mM phosphate buffer, pH 7·4, 3 mM oxidized glutathione, 2 mM EDTA, 83 μM NADPH, rat liver cytosolic fraction (3 ml total volume). DCVC and DCVMP were either added immediately to the assay system (20 μl rat liver cytosolic fraction $\equiv$ 0·2 mg protein, taken for the assay) or were preincubated with a sample of cytosolic fraction ($\equiv$ 16·5 mg protein) for 30 min at 37° on a metabolic shaker and 50 μl of this fraction ($\equiv$ 0·51 mg protein) taken for the assay of glutathione reductase.

Assay of DCVC breakdown. DCVC degradation was measured by the production of pyruvic acid and ammonia by the cytosolic fraction of rat liver in the following medium: 67 mM phosphate buffer, pH 7·4; 1 mM DCVC and cytosolic fraction equivalent to 2·0–2·5 mg rat liver protein (3 ml total volume). Ten min preincubation of the medium was followed by the addition of cytosolic fraction. Reaction vessel was sealed and incubated on a metabolic shaker for 30 min at 37°. The reaction was terminated by the addition of ice-cold perchloric acid; pyruvate and ammonia were assayed as already described.[6] Lactate was assayed spectrophotometrically at 340 nm and 25° on a Unicam SP-800 according to the method of Bergmeyer[10] using lactate dehydrogenase (EC 1.1.1.27).

Protein estimation. Protein was estimated by the biuret reaction according to Robinson and Hogden[11] as modified by Aldridge.[12]

TABLE 1. EFFECT OF DCVC AND DCVMP UPON LIPOYL DEHYDROGENASE ACTIVITY OF RAT LIVER MITOCHONDRIA *in vitro*

No preincubation		Preincubation (30 min)	
Control	Control rate (%) 100 (2) [$k = 6{\cdot}4 \times 10^{-2}$ min^{-1}]	Control	Control rate (%) 100 (3) [$k = 1{\cdot}9 \times 10^{-1}$ min^{-1}]
DCVC (200 μM)	119(1)	DCVC (200 μM)	25(3)
DCVC (500 μM)	119(1)	DCVC (200 μM) + semicarbazide (200 μM)	41(1)
DCVMP (100 μM)	91(1)	DCVC (200 μM) + semicarbazide (500 μM)	58(1)
DCVMP (200 μM)	75(1)	DCVMP (200 μM)	79(2)
DCVMP (500 μM)	75(1)	DCVMP (500 μM)	79(2)

Lipoyl dehydrogenase activity was assayed as described in the Materials and Methods section (3 ml total volume). Enzyme activities have been calculated as first order rate constants and expressed as percentage of control rate (number of measurements in parentheses). Measurements of enzyme activity involving no preincubation of inhibitor with supernatant fraction of rat liver mitochondria were performed at 25° and those involving preincubation were performed at 37°. Details of the preincubation of inhibitor with supernatant fraction of rat liver mitochondria are presented in the Materials and Methods section.

Special reagents. The following reagents were obtained from the sources indicated: oxidized lipoic acid, Sigma Chemical Co. NADH, NADPH, NAD, oxidized glutathione and lactate dehydrogenase, Boehringer & Soehne. DCVC and DCVMP were synthesized as described by Parker.[4]

RESULTS

Lipoyl dehydrogenase the only enzyme common to both pyruvate and 2-oxoglutarate dehydrogenase catalyses the reversible reduction of lipoic acid using NADH as hydrogen donor. Comparison has been made of the effects of DCVC and DCVMP upon lipoyl dehydrogenase activity by; (1) the immediate addition of DCVC or DCVMP to the assay system and; (2) preincubation of DCVC or DCVMP with a sample of mitochondrial supernatant fraction from disrupted mitochondria prior to assay (this fraction has the capacity to degrade DCVC to an active inhibitor[6] and possesses lipoyl dehydrogenase activity). The effects of the immediate addition of DCVC or DCVMP upon lipoyl dehydrogenase are shown in Table 1. Concentrations of DCVC and DCVMP which are effective against respiration of isolated rat liver mitochondria have been employed. Whilst 100–500 μM DCVMP cause slight inhibition of lipoyl dehydrogenase activity, DCVC, at both concentrations tested (200, 500 μM) causes a slight increase in enzyme activity. However, if DCVC or DCVMP is preincubated (as described in the Methods section) prior to assay of lipoyl dehydrogenase, a striking difference in the effects of the two compounds is observed. Table 1 shows that whilst DCVMP causes slight inhibition of enzyme activity, DCVC causes a marked inhibition. It is therefore evident that DCVMP has no significant action upon lipoyl dehydrogenase *in vitro*. Since DCVC only inhibits after preincubation of inhibitor with supernatant fraction, it is suggested that metabolism of DCVC to an active inhibitor is required before inhibition develops. The metabolism of DCVC by rat liver mitochondria can be partially blocked by semicarbazide,[6] and furthermore semicarbazide and DCVC preincubated with mitochondrial supernatant fraction for 30 min at 37° on a metabolic shaker will partially prevent the inhibition of lipoyl dehydrogenase activity by DCVC (Table 1).

Support for the view that DCVC and DCVMP produce their inhibitory actions through different mechanisms has been obtained with *in vitro* studies on rat liver

TABLE 2. MEASUREMENT OF PYRUVIC ACID AND AMMONIA FORMED FROM DEGRADATION OF DCVC BY THE CYTOSOLIC FRACTION OF RAT LIVER

Time (min)	Pyruvate (nmole/mg protein)		Lactate (nmole/mg protein)		Ammonia (nmole/mg protein)	
	Control	DCVC	Control	DCVC	Control	DCVC
10		15·0 ± 2·1 (5)		14·8 (1)		
20		27·1 ± 3·3 (5)		22·8 (1)		
30	7·7 (2)	40·9 ± 3·2 (8)	6·0 (2)	29·1 ± 4·9 (5)	0 (2)	66·5 (2)

Results are expressed as mean ± S.E. (number of observations in parentheses).

Each assay for DCVC breakdown contained 67 mM phosphate buffer, pH 7·4, 1 mM DCVC, and the cytosolic fraction of rat liver (≡ 2·0–2·5 mg protein). Total volume 3 ml. Reaction temperature was 37° and the assay was performed after 10 min preincubation of medium. Pyruvate and ammonia were estimated as described under Materials and Methods.

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TABLE 3. EFFECT OF DCVC AND DCVMP UPON GLUTATHIONE REDUCTASE ACTIVITY OF RAT LIVER CYTOSOL *in vitro*

No preincubation		Preincubation (30 min)	
Control	Control rate (%) 100 (2) [$k = 1{\cdot}3 \times 10^{-1}$ min^{-1}]	Control	Control rate (%) 100 (3) [$k = 6{\cdot}8 \times 10^{-1}$ min^{-1}]
DCVC (200 μM)	108 (1)	DCVC (100 μM)	75 (1)
DCVMP (200 μM)	115 (1)	DCVC (1mM)	25 (2)
		DCVMP (100 μM)	104 (1)
		DCVMP (1mM)	107 (2)

Glutathione reductase activity was assayed as described in the Materials and Methods section (3 ml total volume). Enzyme activities have been calculated as first order rate constants and expressed as percentage of control rate (number of measurements in parentheses). All measurements of enzyme activity were made at 37°. Details of the preincubation of inhibitor with rat liver cytosol are presented in the Materials and Methods section.

glutathione reductase. Glutathione reductase catalyses the reduction of oxidized glutathione by reduced NADP and the enzyme contains an active site configuration similar to lipoyl dehydrogenase.[13,14] In order to study the effect of DCVC upon glutathione reductase, it has been necessary to determine whether the cytosolic fraction of rat liver has any capacity to metabolize DCVC to pyruvate, ammonia and an active inhibitor. The results of Table 2 show that rat liver cytosol can metabolize DCVC to pyruvate (+ lactate) and ammonia. Since the cytosol contains the capacity to degrade DCVC, the approach to the study of glutathione reductase activity has been similar to that for lipoyl dehydrogenase activity.

Table 3 shows that direct addition of DCVC or DCVMP to the assay system causes a slight increase in glutathione reductase activity. However, preincubation of DCVC or DCVMP with rat liver cytosol prior to assay shows that whilst DCVMP does not affect the activity of glutathione reductase, DCVC markedly inhibits the activity (Table 3).

DISCUSSION

Rat liver has the capacity to degrade DCVC to pyruvic acid and ammonia. This capacity has a dual localization. Mitochondrial[6] and cytosolic fractions isolated from rat liver have the ability to degrade DCVC to pyruvic acid and ammonia in a 1:1 stoichiometry and at least one other unidentified metabolite, which has the characteristics of an alkylating agent.[15] Hydrolysis of DCVC by enzymes from several tissues produces pyruvate, ammonia, chloride ions and a metabolite containing the thiovinyl grouping in the presence of an acceptor for the metabolite.[16]

In a previous paper[5] it was shown that DCVC and DCVMP inhibited the 2-oxoacid dehydrogenases of rat liver mitochondria *in vitro*. Since the 2-oxoacid dehydrogenases are multi-enzyme complexes possessing the same cofactor requirements, a study has been made of the potential component(s) in the complexes with which DCVC and DCVMP combine. Lipoyl dehydrogenase, an enzyme present in both complexes and glutathione reductase, an extramitochondrial enzyme have similar properties; (1) they catalyse similar reactions; (2) both are flavoproteins possessing similar active site configurations.[17] The activities of both enzymes are not inhibited by the immediate addition of DCVC to the assay systems but they are after preincubation. These observations are of two-fold interest in that; (i) the site of action of DCVC is indicated and; (ii) the metabolism of DCVC to an active inhibitor is required before inhibition develops; furthermore, the absence of membrane barriers in the rat liver fractions eliminates any permeability problems. Although the cytosol of rat liver has a greater capacity than liver mitochondria[6] to metabolize DCVC to pyruvic acid and ammonia, the inhibition of lipoyl dehydrogenase is more powerful than that of glutathione reductase. The inability to inhibit glutathione reductase so strongly may be related to the presence of glutathione in rat liver cytosol for which the thiovinyl moiety of DCVC is known to have affinity.[18] Additionally, rat kidney, the major target organ for DCVC *in vivo* has the ability to degrade DCVC to pyruvic acid and ammonia and inhibition of glutathione reductase can be demonstrated *in vitro*.[15]

Many observations have suggested that DCVC requires metabolism before inhibition develops. DCVC produces a delayed inhibition of respiration and a delayed stimulation of ATP hydrolysis by rat liver mitochondria,[5] and DCVC does not

immediately inhibit lipoyl dehydrogenase and glutathione reductase activities of rat liver.

Lipoyl dehydrogenase and glutathione reductase catalyse the reversible reduction of lipoic acid and glutathione respectively using NADH and NADPH as hydrogen donor respectively. The flavoprotein enzymes contain 2 moles FAD/mole enzyme. Although two electrons per flavin are donated to the enzyme, it has been shown that the flavin only accepts one electron; another group (a redox active disulphide) accepts the other electron.[13,17] The two enzymes function catalytically between the fully oxidized and semiquinone states.

It is suggested therefore that a metabolite formed from DCVC (which may be 1,2-dichlorovinyl mercaptan) interacts with loss of chlorine atoms with the configuration common to both enzymes (FAD and the redox active disulphide).

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