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# THE METABOLISM OF S-(1,2 DICHLOROVINYL)-L-CYSTEINE BY RAT LIVER MITOCHONDRIA

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**Abstract**—Isolated rat liver mitochondria degrade DCVC to pyruvic acid and ammonia. Another metabolite of unknown structure qualitatively possesses the characteristics of an alkylating agent. Pyridoxal 5′-phosphate catalyses the non-enzymic breakdown of DCVC to pyruvic acid and ammonia. Semicarbazide, a pyridoxal 5′-phosphate trapping reagent blocks the metabolism of DCVC by rat liver mitochondria. This metabolism of DCVC by rat liver mitochondria is judged to be relevant to the inhibition of respiration of rat liver mitochondria which is delayed in onset and which was reported in the preceding paper.

IN A PREVIOUS paper, evidence has been presented to indicate that the site of action of S-(1,2 dichlorovinyl)-L-cysteine [DCVC] and S-(1,2 dichlorovinyl)-3-mercaptopropionic acid [DCVMP] in rat liver mitochondria is the 2-oxoacid dehydrogenases.[1]

The time course and form of inhibition of respiration of rat liver mitochondria by the two compounds is distinctly different *in vitro*; DCVMP produces an immediate inhibition whilst DCVC causes a progressive inhibition which is delayed in onset. A possible interpretation of this delay is that DCVC may have to be metabolised to an active inhibitor.

Cleavage of DCVC at the C—S bond has already been shown to occur enzymically in bovine liver and kidney extracts[2] and non-enzymically.[3] Products identified from these cleavage reactions are pyruvic acid, ammonia and chloride ions; another metabolite of unknown structure containing the thiovinyl moiety of DCVC is also a product.

This paper shows that DCVC can be degraded by rat liver mitochondria *in vitro* and the relevance of this observation to its intramitochondrial site of action is discussed.

## MATERIALS AND METHODS

### *Preparation of homogenate*

Ten per cent homogenate of rat liver was prepared in 67 mM phosphate buffer pH 7·4.

### *Isolation of mitochondria*

Rat liver mitochondria were prepared from whole liver in 300 mM sucrose as described by Aldridge.[4]

*Measurement of respiration*

Oxygen uptake was measured manometrically according to Aldridge.[5] The basic reaction medium for mitochondria had the following composition: 100 mM KCl, 14 mM $MgCl_2$, 1 mM EDTA, 16·7 mM potassium phosphate, pH 6·8 (3 ml total vol.) and the reaction temperature was 37°. Substrate and adenine nucleotide were added as described in the legends.

*Measurement of pyruvic acid*

Pyruvic acid was estimated according to Bergmeyer[6] (with lactic dehydrogenase, EC 1.1.1.27) in the supernatant remaining after acid precipitation of mitochondria or homogenate and subsequent neutralization.

*Measurement of ammonia*

Ammonia was collected by the microdiffusion method of Conway[7] and determined spectrophotometrically with Nessler's reagent.

*Isolation of subfractions of rat liver mitochondria*

Isolated rat liver mitochondria were fractionated by swelling, shrinking, sonication and discontinuous density gradient centrifugation according to Sottocasa and co-workers.[8] Monoamine oxidase activity was assayed spectrophotometrically at 25° according to Schnaitman and co-workers,[9] by measuring the production of benzaldehyde at $E_{250}$ nm. NADH oxidase activity was assayed polarographically at 37° in a medium containing: 67 mM phosphate buffer pH 7·4, 2 mM NADH, 10 $\mu$m cytochrome c (total vol. 3 ml). After measurement of total NADH oxidation, 1·1 $\mu$m rotenone was added and residual NADH oxidation determined. Rotenone-sensitive NADH oxidase was the difference between these two values.

*Special reagents*

The following reagents were obtained from the Sigma Chemical Co: ATP, (disodium salt from equine muscle), NADH, (disodium salt), cytochrome c, (from horse heart Type III), sodium pyruvate (Type II), pyridoxal 5'-phosphate. 4-(*p*-nitrobenzyl)-pyridine, rotenone, hydroxylammonium chloride and semicarbazide hydrochloride were obtained from British Drug Houses; Antimycin A from Boots Pure Drug Co.; Lactic dehydrogenase (from rabbit muscle) from Boehringer and Soehne.

DCVC and DCVMP were synthesised as described by Parker.[10]

*Protein estimation*

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

*Statistics*

Tests of significance were made using the Students *t*-test.

## RESULTS

Since inhibition of respiration of rat liver mitochondria by DCVC added *in vitro* and by administration of DCVC to the intact animal has already been demonstrated,[1]

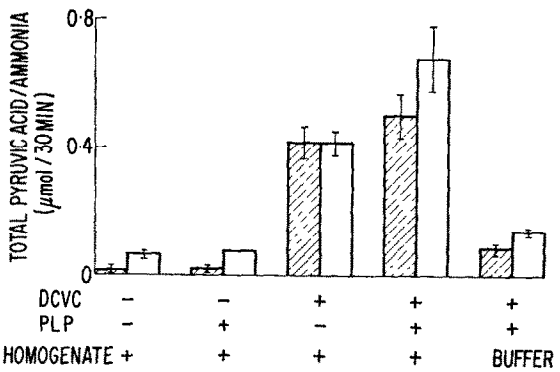


FIG. 1. Degradation of DCVC (i) non-enzymically and (ii) by rat liver homogenate. Each assay contained 67 mM phosphate buffer pH 7·4 and where appropriate, 4·6 mM DCVC, 0·8 mM pyridoxal 5′-phosphate (PLP), 1% rat liver homogenate in buffer in a total volume of 3 ml. Reaction stopped with perchloric acid after 30 min incubation at 37°. Pyruvate and ammonia determined as described under methods.

▨ Pyruvic acid, □ Ammonia, I Range of data.

rat liver has been further investigated for its ability to degrade DCVC. Figure 1 shows that DCVC can be degraded to pyruvic acid and ammonia by rat liver homogenate. The two metabolites are formed in approximately equimolar amounts over 30 min. By inference from the structure of DCVC, at least one other metabolite containing the dichlorothiovinyl moiety of DCVC must be formed in this degradative process.

Many amino-acid derivatives which possess an electronegative $\beta$ substituent are subject to non-enzymic $\beta$ elimination reactions in which the products formed are the corresponding oxo-acid, ammonia and the $\beta$ substituent.[13–15] Such reactions are catalysed by pyridoxal or its 5′-phosphate derivative. Figure 1 shows that pyridoxal 5′-phosphate catalyses a nonenzymic breakdown of DCVC to pyruvic acid and ammonia; more ammonia than pyruvic acid is liberated under these conditions. Some increase in the extent of breakdown of DCVC by rat liver homogenate is brought about by the addition of pyridoxal 5′-phosphate to homogenate.

If the amounts of pyruvic acid and ammonia generated non-enzymically are added respectively to those amounts produced in the homogenate in the absence of exogenous pyridoxal 5′-phosphate and compared with the amounts of pyruvic acid and ammonia produced from DCVC in the homogenate in the presence of exogenous pyridoxal 5′-phosphate, it may be deduced that breakdown of DCVC is due to homogenate. The amounts of pyruvic acid and ammonia measured may not accurately reflect the extent of breakdown since both of the common intermediary metabolites formed from DCVC breakdown can be further utilised by rat liver homogenate.

Rat liver mitochondria have been tested for their ability to degrade DCVC, because of the delayed onset of inhibition of respiration observed. The amounts of pyruvic acid and ammonia produced from DCVC by rat liver mitochondria are shown in Tables 1 and 2. DCVC is degraded by rat liver mitochondria to pyruvic acid and ammonia in a 1:1 stoichiometry. Restriction of pyruvate utilisation by rat liver mitochondria has been achieved by using a relatively high concentration of DCVC, since the latter inhibits pyruvate dehydrogenase of rat liver mitochondria.[1] To confirm the extent of breakdown of DCVC by rat liver mitochondria, pyruvate has been

TABLE 1. MEASUREMENT OF PYRUVIC ACID FORMED FROM THE DEGRADATION OF DCVC BY ISOLATED RAT LIVER MITOCHONDRIA

| Time (min) | Pyruvic acid production (nmole/mg protein) | | | | |
|---|---|---|---|---|---|
| | Control (no DCVC) | DCVC | DCVC + Antimycin | DCVC + Phenyl arsenious acid | DCVMP |
| 10 | 1·8 ± 0·6 (4) | 27·5* ± 3·9 (4) | — | — | 2·4 ± 0·9 (5) |
| 20 | 2·5 ± 0·6 (4) | 56·5* ± 8·3 (4) | — | — | 5·4 ± 1·8 (5) |
| 30 | 5·2 ± 1·2 (5) | 69·0* ± 4·6 (6) | 75·2 (2) | 58·5 (2) | 5·6 ± 1·9 (5) |

Results expressed as mean ± S.E. with number of observations in parentheses.
* $P < 0.001$ with respect to control.

Each assay contained 3 ml basic reaction medium, pH 6·8. Additions were 2·3 mM ATP, rat liver mitochondria (3–4 mg protein) in 30 mM sucrose final and where appropriate 1 mM DCVC, 1 mM DCVMP, 3·6 μg antimycin A, 18 μM phenylarsenious acid. Reaction temperature was 37° and assays performed after 10 min preincubation of medium. Pyruvate was estimated as described under methods.

measured in the presence of an immediate inhibitor of pyruvate dehydrogenase activity, phenylarsenious acid, at a concentration which totally suppresses pyruvate oxidation. The amounts of pyruvic acid detected are slightly lower in the presence of phenylarsenious acid (Table 1). Since pyruvate cannot be converted to lactate by rat liver mitochondria and its oxidation is blocked, then only 7–8% of DCVC added to rat liver mitochondria is broken down by the mechanism which yields pyruvic acid and ammonia. Also shown in Table 1 is the effect of antimycin A upon degradation of DCVC to pyruvate. Antimycin A, which inhibits $NAD^+$ and FP-linked respiration of rat liver mitochondria (inhibits between cytochromes b and c of the mitochondrial respiratory chain) does not alter the extent of DCVC breakdown at a concentration which completely blocks mitochondrial respiration.

No direct involvement of pyridoxal 5′-phosphate in the degradation of DCVC by rat liver mitochondria has been demonstrated but with the aid of agents which inactivate pyridoxal 5′-phosphate by combination with the aldehyde group, it has been

TABLE 2. MEASUREMENT OF AMMONIA FORMED FROM THE DEGRADATION OF DCVC BY ISOLATED RAT LIVER MITOCHONDRIA

| Ammonia production (nmole/mg protein/30 min) | | | |
|---|---|---|---|
| Control (No DCVC) | DCVC | Control + semicarbazide | DCVC + semicarbazide |
| 4 ± 0·9 (3) | 61* ± 4·5 (5) | 11·5 (2) (11·0, 12·0) | 12 ± 2·5 (6) |

Results expressed as mean ± S.E. with number of observations in parentheses.
* $P < 0.001$ with respect to control.

Each assay contained 1 ml basic reaction medium. Additions were 2·3 mM ATP rat liver mitochondria (1–1·3 mg protein) in 30 mM sucrose final and where appropriate 1 mM DCVC, 1 mM semicarbazide. Reaction temperature was 37° and assays performed after 10 min preincubation of medium. Ammonia was estimated as described under methods.

possible to study the degradation of DCVC in the presence of such agents, e.g. hydroxylamine, semicarbazide. These agents have been of limited value only, since (i) they are not specific reagents (ii) pyruvate cannot be measured in their presence since they combine reversibly with the carbonyl group of pyruvic acid (iii) hydroxylamine can be degraded to ammonia by rat liver mitochondria.[16] Addition of semicarbazide to rat liver mitochondria in the presence of DCVC shows that semicarbazide prevents the formation of ammonia from DCVC (Table 2). Semicarbazide itself yields small amounts of ammonia in this system.

In this study semicarbazide has been employed in the mitochondrial system to assess the alkylating potentials of DCVC and the moiety of unknown structure derived from it. The use of 4-(*p*-nitrobenzyl)-pyridine [NBP] as a chemical acceptor for alkyl groups is widely recognised.[17] Using the non-aqueous method of these authors, we have shown qualitatively that DCVC and DCVMP are potential alkylating agents since both will alkylate NBP to give the characteristic colour reaction.

This colour reaction has been studied in the presence of rat liver mitochondria. Addition of 4·6 mM DCVC and 100 $\mu$M NBP to rat liver mitochondria in the basic reaction medium at 37°, (lightly shaken) leads to the characteristic colour production. This colour formation can be largely prevented by the addition of 1 mM semicarbazide and totally by 1 mM hydroxylamine added initially. Colour production is not destroyed if these reducing reagents are added terminally. These observations are qualitative since the coloured product, which is found to be associated with the mitochondrial pellet after centrifugation, could not be quantitatively extracted with various solvents. DCVMP does not participate in a colour reaction under the conditions described for DCVC and therefore appears to possess no alkylating potential in the mitochondrial system.

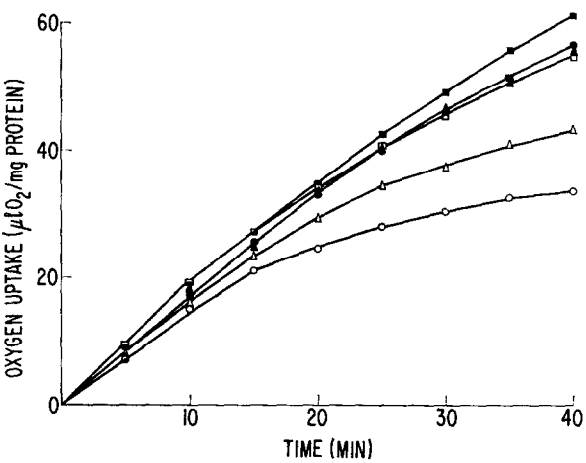


FIG. 2. Effect of semicarbazide on inhibition of DNP-stimulated respiration of rat liver mitochondria by DCVC. Each flask contained 3 ml basic reaction medium, pH 6·8. Additions were 10 mM pyruvate, 1 mM fumarate, 2·3 mM ATP, 30 $\mu$M DNP, rat liver mitochondria (3–4 mg protein) in 30 mM sucrose final and where appropriate 100 $\mu$M DCVC and either 1 or 10 mM semicarbazide (S.C.). Medium and mitochondria preincubated for 10 min at 37° before the addition of DCVC.

■ Control + 10 mM S.C., △ DCVC + 1 mM S.C., □ DCVC + 10 mM S.C., ● Control, ▲ Control + 1 mM S.C., ○ DCVC.

TABLE 3. DEGRADATION OF DCVC AND DISTRIBUTION OF ENZYMES IN SUB-FRACTIONS DERIVED FROM ISOLATED RAT LIVER MITOCHONDRIA

| | Pyruvate (nmole/30 min/ mg protein) | Total pyruvate (μmole/30 min) | Protein range (mg) | NADH oxidase (μgatom O/min/ mg protein) | Total | Monoamine-oxidase ($E^{1cm}_{250}$/min/mg protein) | Total |
|---|---|---|---|---|---|---|---|
| Before centrifugation | 67·9 (Taken from Table 1) | 15·6 | 230–236 | 0·180 | 42·5 | 0·031 | 7·19 |
| | Intact isolated mitochondria | | | Mitochondria after swelling, shrinking and sonication | | | |
| After centrifugation | | | | | | | |
| Heavy fraction | 28·1 | 2·2 | 67–78 | 0·346 | 23·2 | 0·023 | 1·54 |
| Light fraction | 6·7 | 0·2 | 30–31 | 0·097 | 3·0 | 0·093 | 2·88 |
| Soluble fraction | 107·2 | 11·9 | 111–116 | 0·052 | 6·0 | 0·016 | 1·86 |
| Sum (heavy + light + soluble) | | 14·3 | | | 32·2 | | 6·28 |
| Recovery (%) | | | | | 76 | | 87 |

Each assay for DCVC breakdown contained 67 mM phosphate buffer pH 7·4, 1 mM DCVC, the appropriate subfraction in a total volume of 3 ml. Reaction temperature was 37° and assay performed after 10 min preincubation of medium. Pyruvate was estimated as described under methods. Marker enzymes were assayed as described under methods. Protein recovery was 93 per cent.

These observations suggest that the metabolism of DCVC by rat liver mitochondria to produce pyruvic acid and ammonia also yields an alkylating moiety. DCVMP which is not metabolized in this way does not produce an alkylating moiety.

The inhibition of DCVC degradation by semicarbazide has allowed measurement of respiration of rat liver mitochondria in the presence of a fixed concentration of DCVC (100 $\mu$M) and varying concentrations of semicarbazide (Fig. 2). With increasing concentration of semicarbazide and with pyruvate as substrate, inhibition by DCVC is reduced suggesting that semicarbazide is delaying the inhibition of respiration by DCVC presumably by limiting its breakdown.

Localization of the site of DCVC breakdown to pyruvic acid has been made by measuring the degradation of DCVC in sub-fractions derived from isolated rat liver mitochondria (Table 3). The soluble fraction has the capacity to degrade DCVC to pyruvic acid. The distribution of two mitochondrial enzyme markers has been followed to ascertain the extent of contamination of this soluble fraction with membrane components. The distribution of NADH oxidase and monoamine oxidase (marker enzymes for inner and outer membranes respectively) is shown in Table 3. NADH oxidase is enriched in the heavy fraction and monoamine oxidase in the light fraction. The soluble fraction is largely devoid of inner membrane components but still considerably contaminated with outer membrane components. The soluble fraction largely represents components found between the two mitochondrial membranes and in the matrix which is completely enclosed by the inner membrane.

## DISCUSSION

This paper demonstrates that DCVC can be degraded by rat liver homogenate to yield pyruvic acid and ammonia. These same two metabolites were products of DCVC degradation by bovine liver and kidney.[2]

Pyridoxal 5'-phosphate has been shown to catalyse the breakdown of DCVC non-enzymically to pyruvic acid and ammonia; no breakdown was observed in the absence of pyridoxal 5'-phosphate. Whether or not the mechanism of the present process involves a $\beta$ elimination cannot be categorically stated since the $\beta$ substituent has not been identified.

In many cases, the demonstration of a non-enzymic reaction involving pyridoxal 5'-phosphate has led to the identification of a similar enzymic reaction involving pyridoxal 5'-phosphate in which the products of the reactions are the same. Although the mechanisms involved may be similar[18] the non-enzymic breakdown of DCVC utilizing pyridoxal 5'-phosphate does not necessarily infer that enzymic breakdown to the same products involves pyridoxal 5'-phosphate.

One criterion by which the relevance of metabolism to a particular problem can be judged is to block the metabolism of the agent under investigation. Semicarbazide, a non-specific pyridoxal 5'-phosphate trapping reagent has been used to assess the relevance of metabolism to this particular problem. Semicarbazide completely prevented the formation of ammonia from DCVC and also reduced the extent of inhibition of respiration of rat liver mitochondria caused by DCVC.

Another observation using semicarbazide has demonstrated the alkylating potentials of DCVC and a metabolite formed from it. When metabolism of DCVC is allowed to proceed normally, extensive colour formation associated with alkylation of NBP

occurs; addition of semicarbazide leads to almost complete loss of colour production. Bhattacharya and Schultze[19] using purified enzymes which have a high substrate specificity for DCVC and which degrade the molecule have shown that NBP does not form a coloured product with pyruvate, ammonia or chloride ions nor in the absence of DCVC. It therefore seems likely that the metabolite of unknown structure has considerable alkylating ability. However, at this stage, it is not known whether alkylating potential is relevant to the mechanism of inhibition of respiration by DCVC. DCVC or a metabolite causes a progressive inhibition of respiration after a delayed onset.[1] Such a progressive inhibition would be consistent with an alkylation reaction in which chemical reaction occurs leading to the formation of a covalent bond.

The limited breakdown of DCVC by rat liver mitochondria to yield stoichiometric quantities of pyruvic acid and ammonia (1:1) has been shown. More specifically, this breakdown occurs in the soluble fraction of rat liver mitochondria and therefore, the site of metabolism of DCVC intramitochondrially must be in the matrix or intermembrane space.

For DCVC to be metabolized and to produce its inhibitory action, it is evident that DCVC must penetrate one or both mitochondrial membranes as the parent molecule. The inner membrane which completely encloses the matrix of the mitochondrion is the barrier which controls the flux of most compounds, especially charged compounds.[20] Under the experimental conditions in this study, i.e. pH 6·8, DCVC would be expected to be largely negatively charged on account of its possession of two ionisable groups, pK (COOH) = 2·5; $pK_2$ ($NH_2$) = 8·2.[21] Therefore the intramitochondrial penetration of DCVC may be influenced by the charge upon the molecule.

DCVC appears to be an unusual compound in that of the metabolites produced, two, pyruvic acid and ammonia are common intermediary metabolites whilst another possesses considerable alkylating ability and inhibits pyruvate dehydrogenase, an enzyme complex which utilizes pyruvic acid, a metabolite formed from DCVC.

Circumstantial evidence in this paper suggests the possible involvement of pyridoxal 5'-phosphate in DCVC breakdown by rat liver and that metabolism of DCVC by rat liver mitochondria is relevant to the inhibition of respiration caused by this agent *in vitro*.

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