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FORMATION OF 2-METHYL-2,4-THIAZOLIDINEDICARBOXYLIC ACID FROM L-CYSTEINE IN RAT TISSUES

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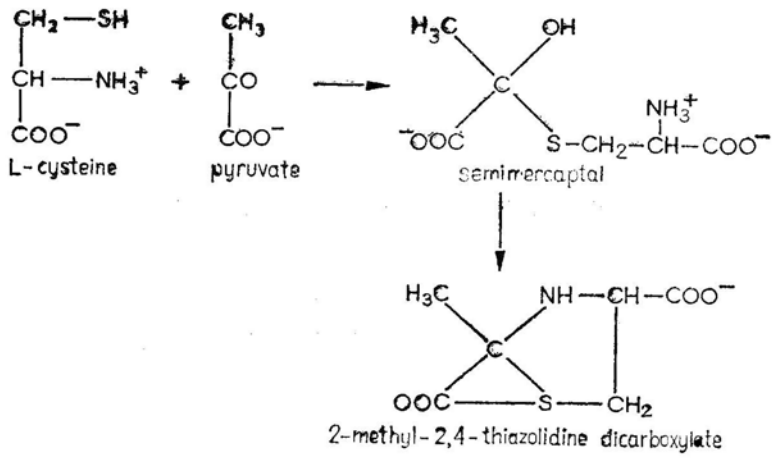
The adduct formed non-enzymatically from L-cysteine and pyruvate: 2-methyl-2,4-thiazolidinedicarboxylic acid (CP) was isolated, and identified by the electron impact mass spectroscopy. It was found that CP is formed (by cysteine transformation) and is metabolized in rat tissues. Formation of CP from cysteine or cystine was catalysed by partially purified rat liver γ-cystathionase.

Disappearance of pyruvate in the presence of cysteine at physiological pH has been repeatedly reported (Brüggemann & Waldschmidt, 1962; Dugajczyk *et al.*, 1968; Guarneros & Ortega, 1970). Some authors (Brüggemann & Waldschmidt, 1962; Dugajczyk *et al.*, 1968) postulated formation *in vivo* of the adducts between cysteine and not precisely identified carbonyl compounds. The possibility of non-enzymatic condensation of cysteine and pyruvate to form 2-methyl-2,4-thiazolidinedicarboxylic acid (CP) was suggested by Schubert (1937), who has synthesized this compound (Scheme 1). Kredich *et al.* (1973) found that purified γ-cystathionase (cystathionine γ-lyase, EC 4.4.1.1) from *Salmonella typhimurium* catalysed formation of CP. In 1977 Simpson & Freedland demonstrated CP synthesis from cysteine in perfused rat liver. The physiological significance of CP remains, however, unexplained. Until now it is not known whether the reaction occurs in the living mammalian tissues, and whether CP is further metabolized.

The reactivity of cysteine with carbonyl compounds and toxicity of cysteine (Harper *et al.*, 1970) prompted us to study formation of CP from cysteine and possibility of its further metabolism in rat tissues.

MATERIALS AND METHODS

Material. Isolated tissues of adult rats, killed by decapitation, were homogenized in 0.1 M-phosphate buffer, pH 7.0 (1:10, w/v). Homogenates were centrifuged for 10 min at 650 *g* and the supernatants were used for CP determination.



Scheme 1. Formation of 2-methyl-2,4-thiazolidinedicarboxylic acid.

Mitochondria isolated from liver according to Schibko *et al.*, (1967) and suspended in 0.1 M-phosphate buffer, pH 7.5, were frozen and thawed three times, homogenized, centrifuged at 15 000 *g* for 30 min and used for the assay.

Reagents. L-Cysteine was produced by r.c.b. (Bruxelles, Belgium), DL-homoserine was from Koch-Light Labs. Ltd (England); L-cystine was purchased from E. Merck (Darmstadt, F.R.G.). CP was synthesized by incubation of 0.1 M-cysteine and 0.1 M-pyruvate in water at 37°C during 2 h. Chromatography of the reaction mixture was performed on Whatman 3 *MM* paper in butanol/acetic acid/water (40:10:10, by vol.). The position of cysteine and the cysteine-pyruvate adduct was detected with ninhydrin reagent (R_F for CP was 0.180). The condensation product was eluted with water and lyophilized. Crystalline, slightly yellow and strongly hygroscopic substance was obtained.

Assay of "free" and "total" pyruvate. The content of "free" pyruvate and "total" pyruvate (the latter comprising both "free" pyruvate and that combined with cysteine in CP) was determined according to Kredich *et al.* (1973) with some modifications: pyruvate 2,4-dinitrophenylhydrazone was extracted with toluene, and 2 ml of 2.5% alcoholic KOH was added to 1.5 ml of the toluene extract. After 10 min the absorbance at 520 nm was measured.

Kinetics of non-enzymatic formation of CP from L-cysteine and pyruvate. Solutions with varying concentration of L-cysteine and pyruvate were assayed for "free" and "total" pyruvate as a function of time. The effect of pH on the non-enzymatic synthesis of CP was studied using 0.02 M Britton-Robinson buffer at the appropriate pH.

Formation and degradation of CP in homogenates from rat tissues. CP formation was calculated from the difference between "total" and "free" pyruvate derived from L-cysteine during its incubation with the tissue extracts. The decrease in CP

concentration in the incubation mixture was taken as the measure of CP degradation. To eliminate the non-enzymatic hydrolysis of CP, the control samples were run without the homogenates.

For the study of CP degradation by mitochondrial extract, CP (10 μmol) was incubated with 0.3 ml (0.5 mg protein) of mitochondrial extracts in a total volume of 0.5 ml. The reaction was stopped at appropriate time intervals by adding 4 ml of 95% ethyl alcohol, and the mixture was centrifuged. The absorbance of the alcoholic supernatant was determined at 250 and 270 nm, and the results were corrected for the absorbance of the control samples containing the mitochondrial extract without the substrate. The absorbance of CP without mitochondrial extract remained unchanged during incubation.

Assay for γ-cystathionase activity. Rat liver cystathionase was isolated according to Greenberg (1962) without the precipitation with protamine sulphate and crystallization steps. Specific activity was determined according to Matsuo & Greenberg (1958) using homoserine as a substrate. The level of 2-oxoacids was determined by the method of Friedemann & Haugen (1943).

Mass spectra were measured on LKB 9000 S spectrometer at 70 eV.

RESULTS AND DISCUSSION

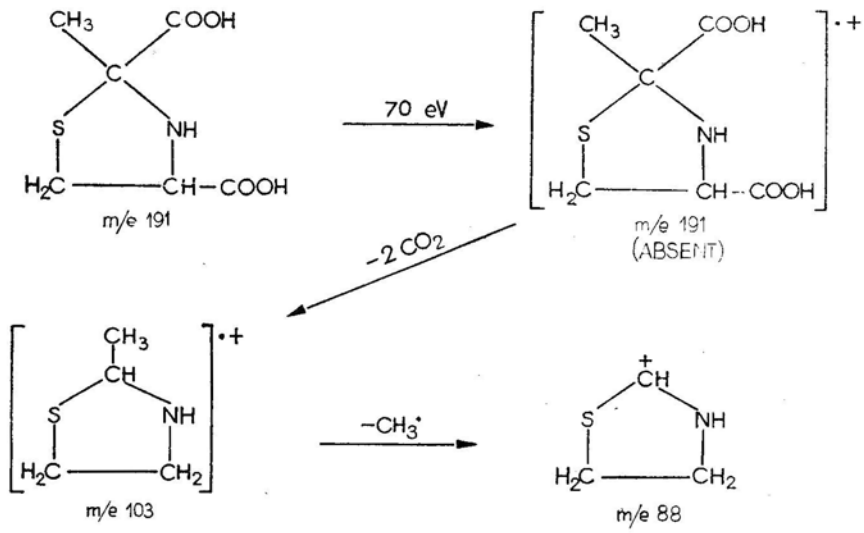
Identification of 2-methyl-2,4-thiazolidinedicarboxylic acid

CP formed non-enzymatically from cysteine and pyruvate was identified by the electron impact mass spectroscopy. As it was expected, the molecular peak was not observed in the CP molecule (Scheme 2) because of the presence of two carboxyl groups. The fragmentation, started by the elimination of two molecules of carbon dioxide, gave a weak fragmentation peak of the radical-ion at mass *m/e* 103 (3.1%), which lost the methyl radical and formed an ion corresponding to thiazolidine, responsible for a visible peak at mass *m/e* 88 (64.4%).

Formation and degradation of CP

Non-enzymatic. As can be seen in Fig. 1, the optimum pH for the non-enzymatic reaction between L-cysteine and pyruvate is at pH from 7.0 to 8.0, whereas the optimum for CP hydrolysis is at pH 9.0. Figure 2 shows the substrate-dependent non-enzymatic formation of CP at pH 7.5.

In rat tissues. There are several metabolic pathways of L-cysteine transformation to pyruvate: in the reaction catalysed by cysteine desulphhydrase or *via* transamination and transsulphuration. It should be expected that synthesis of CP from L-cysteine *in vivo* will result from the tissue capability for both L-cysteine transformation to pyruvate and its subsequent reaction with the remaining L-cysteine to form CP. As shown in Table 1, CP synthesis from L-cysteine in rat tissues is most intense in skeletal muscles, then liver and kidney. On the other hand, cysteine desulph-



Scheme 2. Fragmentation of 2-methyl-2,4-thiazolidinedicarboxylic acid.

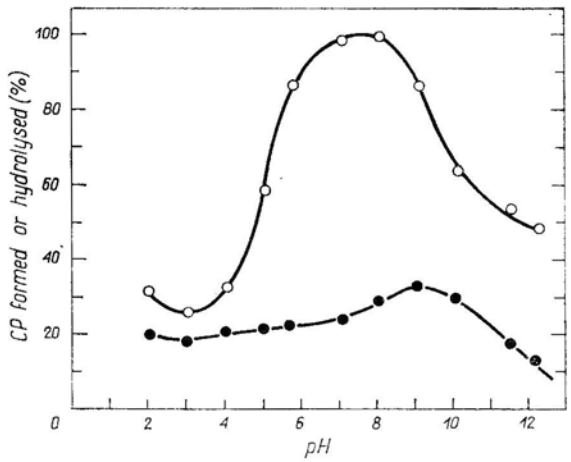


Fig. 1. Effect of pH on the non-enzymatic synthesis (○) and degradation (●) of 2-methyl-2,4-thiazolidinedicarboxylic acid (CP). CP was assayed as described under Materials and Methods. The reaction mixture for CP formation contained in a final volume of 1.0 ml : 10 μmol of L-cysteine, 1 μmol of pyruvate in 0.02 M Robinson-Britten buffer at the pH indicated. The reaction mixture for CP hydrolysis contained in a final volume of 1.0 ml: 1.0 μmol of CP in Britton-Robinson buffer at the pH indicated. The incubation time in both cases was 1 h.

hydrase activity reported by Koj & Frendo (1962) was the highest in liver and lower in kidney and muscles. Thus, no relation is observed between the activity of this enzyme and CP formation.

On the other hand, degradation of CP was most intense in heart muscle, then in skeletal muscle and liver (Table 1). The results obtained lead to the conclusion that CP is formed and may undergo further transformation in rat tissues at different rates.

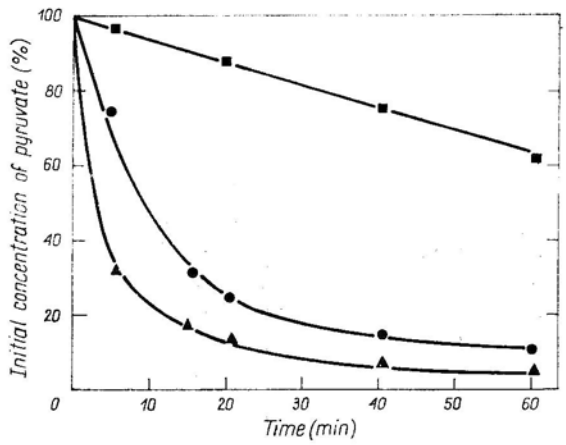


Fig. 2. Time-course of non-enzymatic reaction of pyruvate with L-cysteine. Solutions of L-cysteine and pyruvate were incubated at 37°C in 0.1 M-phosphate buffer (pH 7.5). The initial L-cysteine concentration was: 15 mM (■), 20 mM (●) and 50 mM (▲), and pyruvate concentration 1.5, 2 and 5 mM, respectively.

More detailed studies were based on spectrometric analysis of the incubation mixture of CP with the liver mitochondrial extract. CP, having a saturated ring, does not show specific absorbance in ultraviolet light. During incubation the absorbance at 250 nm was very distinctly increased, especially rapidly within the first 15 min of incubation, and reached its maximum after 2 h (Fig. 3). A smaller increase in absorbance at 270 nm was also observed. This suggests formation of a thiazoline derivative, since the maximum absorbance of thiazoline (Weisiger et al., 1955; Basford & Huenneckens, 1955) is at 245 - 250 nm, with the second minute peak at 275 nm. Therefore one may assume that oxidation of the thiazolidine ring of CP into thiazoline derivative constitutes the first step of CP transformation.

CP synthesis by cystathionase. In further investigations we have proved that CP formation is catalysed by γ-cystathionase (EC 4.4.1.1). The increasing ability of cystathionase preparations to synthesize CP from cysteine was evidenced during purification of the enzyme (Table 2A). On the other hand, the ability for CP metabolism observed in the crude homogenate, disappeared in the purified preparations (Table 2B). As it was reported (Binkley, 1950; Matsuo & Greenberg, 1958; Cavallini *et al.*, 1962), cystathionase is identical with cysteine desulphhydrase, and acts not on cysteine but on cystine which is always present in cysteine solution (Cavallini *et al.*, 1962). CP was found to be a product of cysteine and also of cystine (Fig. 4). This fact explains the observed inconsistencies in cystathionase kinetics. Matsuo & Greenberg (1958), Cavallini *et al.* (1960), Flavin & Segal (1964) and Mazelis *et al.* (1967) observed that formation of 2-oxoacids from cysteine, cystine and cystathionine catalysed by cystathionase was non-linear and lower as compared with formation of other reaction products, such as H_2S or NH_3. The discrepancies observed have been so far attributed to the inhibiting effect of cysteine on the enzyme

Table 1

Formation of 2-methyl-2,4-thiazolidinedicarboxylic acid (CP) from L-cysteine, and degradation of CP in rat tissues

CP was assayed as described under Materials und Methods. The reaction mixture for CP formation contained in a final volume of 1.0 ml: 50 µmols of cysteine and 0.5 ml of homogenate (1.2—4.8 mg protein) in 0.1 M-phosphate buffer, pH 7.5. The reaction mixture for CP degradation contained in a final volume of 1.0 ml: 10 µmol of CP and 0.5 ml of homogenate in 0.1 M-phosphate buffer, pH 7.0. The incubation time in both cases was 2 h. The results (nmol/mg protein) are mean values of five experiments; the limit values are given in parentheses.

Tissue	CP formation	CP degradation	Tissue	CP formation	CP degradation
Kidney	10.7± 0.98 (9.6-12.3)	7.48± 0.79 (6.39- 8.63)	Lung	9.37±0.34 (8.91-9.68)	5.52±0.39 (5.19-6.19)
Liver	12.6± 1.21 (10.9-14.0)	9.41± 0.47 (8.98- 9.91)	Stomach	0.0	0.0
Skeletal muscle	18.14± 1.71 (15.81-19.46)	14.88± 1.16 (13.47-16.68)	Spleen	0.0	0.0
Heart muscle	5.87± 0.58 (5.41- 6.67)	17.83± 1.33 (16.7-21.0)			

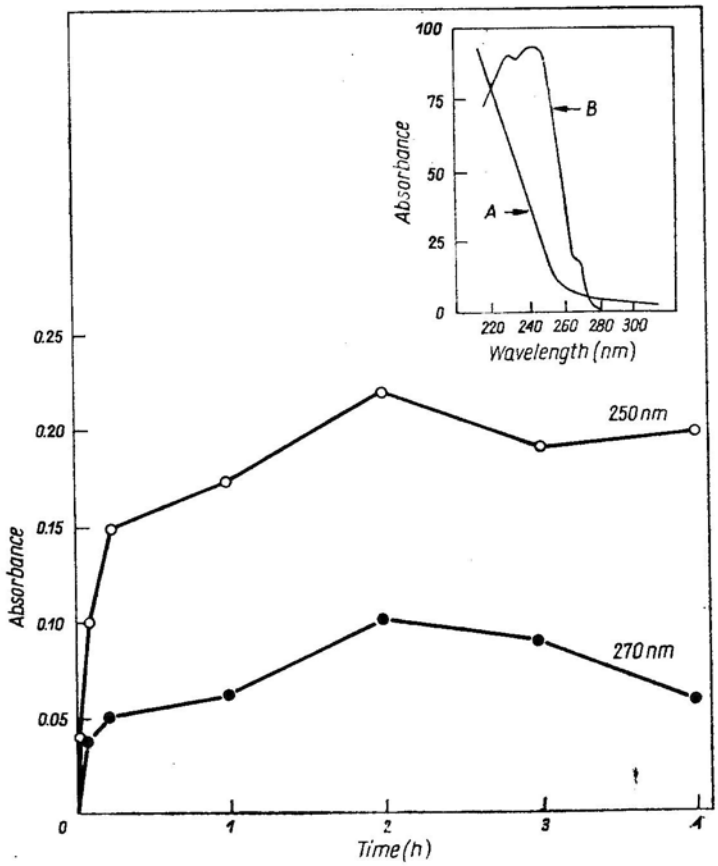


Fig. 3. Changes in absorbance at 250 nm (○) and 270 nm (●) during incubation of CP with rat liver mitochondria. The absorbance of the mitochondrial extract without substrate has been subtracted. Absorbance of *A*, thiazolidinedicarboxylic acid; *B*, 2-methylthiazoline (Basford & Huennekens, 1955) is given in the insert.

(Fernandez & Horvath, 1963; Mazelis *et al.*, 1967) but our results suggest that they may be explained by the interaction of 2-oxoacids with cysteine to form thiazolidine derivatives. Nevertheless, inhibition of cystathionase by cysteine *via* its interaction with pirydoxal phosphate carbonyl group cannot be excluded (Buell & Hansen, 1960; Meister, 1965).

Physiological significance of thiazolidine derivatives. Synthesis and metabolism of some other adducts of cysteine and carbonyl compounds were also reported. Cavallini *et al.* (1956) and Mackenzie & Harris (1957) demonstrated that thiazolidine carboxylic acid, the cysteine-formaldehyde adduct, is oxidized in liver to *N*-formylcysteine. Fitzpatrick & Massey (1982) reported that thiazolidine dicarboxylic acid, the cysteamine-glyoxylate adduct, is a substrate for D-amino acid oxidase, and that thiazoline-2-carboxylate, the suspected physiological product of the reaction of the D-amino acid oxidase, is the inhibitor of dopamine β-hydroxylase (Naber *et al.*,

Table 2

CP formation from cysteine (A) and CP degradation (B) at the successive steps of cystathionase purification

In A, the reaction mixture contained in a final volume of 1.0 ml: 10 μmol of L-cysteine in 0.05 M-phosphate buffer, pH 7.5, and the enzyme fractions (0.34 - 6.6 mg protein). In B, the reaction mixture contained: 10 μmol of CP in 0.05 M-phosphate buffer, pH 7.5, and the enzyme fractions (0.34 - 6.6 mg protein) in a final volume of 1 ml. Both in A and B the mixtures were incubated for 30 min at 37°C.

Purification step	Specific activity	A CP formation (nmol/mg protein)	B CP degradation nmol/mg protein)
Crude homogenate	0.55	13.0	22.1
Heating (60°C, 5 min)	1.23	29.0	11.3
Ammonium sulphate (0.55 - 0.75 sat.)	6.3	42.1	0.0
Ethanol (49% concn.)	27.3	125.0	0.0

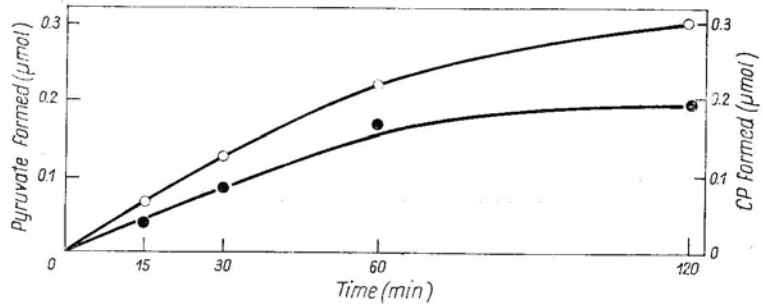


Fig. 4. Formation of pyruvate (○) and CP (●) from cystine by the partially purified cystathionase. The reaction mixture contained in the final volume of 1 ml : 0.1 ml of 0.3 M-cystine solution in 1 M-HCl, 0.1 ml of 1M-bicarbonate solution, 0.5 ml of 0.1 M-phosphate buffer, pH 7.5; the enzyme preparation (ethanol fraction) was added at a concentration of approximately 0.05 unit. Incubation for 30 min at 37°C. Assay conditions as described in the text.

1982). Also a thiazolidine derivative is formed in detoxication of cyanide by cystine (Wood & Cooley, 1956).

Formation and metabolism of thiazolidine derivatives in the metabolism of carbonyl compounds seems to be of interest. CP formation may explain the physiological significance of this type of adduct. It is well known that even moderate doses of cysteine are highly toxic to experimental animals (Birnbaum *et al.*, 1957). The cause of cysteine toxicity is still unknown, however it might be suspected that trapping by cysteine of essential carbonyl compounds may be partially responsible for this toxicity.

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SYNTEZA KWASU 2-METYLO-2,4-TIAZOLIDYNODWUKARBOKSYLOWEGO Z L-CYSTEINY W TKANKACH SZCZURA

Streszczenie

Wyizolowano i zidentyfikowano metodą spektroskopii masowej kwas 2-metylo-2,4-tiazolidynodwukarboksylowy (CP), produkt nieenzymatycznej reakcji L-cysteiny z pirogronianem. Wykazano, że związek ten powstaje z L-cysteiny i ulega dalszym przemianom w tkankach szczura. CP powstaje również z cysteiny i cystyny w reakcji katalizowanej przez częściowo oczyszczoną cystationazę wątroby szczura

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