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Jun Ohta* Toshihiko Ubuka†

*Okayama University,

†Okayama University,

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Abstract

It has been assumed that the in vivo reduction of 3-mercaptopyruvate, an intermediate of cysteine metabolism, to 3-mercaptolactate is catalyzed by lactate dehydrogenase (EC 1.1.1.27) though no definitive evidence has been presented. In order to examine this assumption, reduction of 3-mercaptopyruvate and its inhibition were studied using rat liver homogenate, lactate dehydrogenase purified from rat liver and anti-lactate dehydrogenase antiserum. Reduction of 3-mercaptopyruvate was actively catalyzed by rat liver homogenate and by the purified lactate dehydrogenase. This reducing activity was completely inhibited by anti-lactate dehydrogenase antiserum. These results indicate that the reduction of 3-mercaptopyruvate to 3-mercaptolactate in rat liver is catalyzed by lactate dehydrogenase.

KEYWORDS: 3-mercaptopyruvate, 3-mercaptolactate, lactate dehydrogenase, antiserum, cysteine metabolism

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Reduction of 3-Mercaptopyruvate in Rat Liver is Catalyzed by Lactate Dehydrogenase

Jun Ohta and Toshihiko Ubuka*

Department of Biochemistry, Okayama University Medical School, Okayama 700, Japan

It has been assumed that the *in vivo* reduction of 3-mercaptopyruvate, an intermediate of cysteine metabolism, to 3-mercaptolactate is catalyzed by lactate dehydrogenase (EC 1.1.1.27) though no definitive evidence has been presented. In order to examine this assumption, reduction of 3-mercaptopyruvate and its inhibition were studied using rat liver homogenate, lactate dehydrogenase purified from rat liver and anti-lactate dehydrogenase antiserum. Reduction of 3-mercaptopyruvate was actively catalyzed by rat liver homogenate and by the purified lactate dehydrogenase. This reducing activity was completely inhibited by anti-lactate dehydrogenase antiserum. These results indicate that the reduction of 3-mercaptopyruvate to 3-mercaptolactate in rat liver is catalyzed by lactate dehydrogenase.

Key words : 3-mercaptopyruvate, 3-mercaptolactate, lactate dehydrogenase, antiserum, cysteine metabolism

3-Mercaptolactate-cysteine disulfide [S-(2-hydroxy-2-carboxyethylthio)cysteine, HCETC] is a constituent of normal human urine (1) and is excreted in large amounts in the urine of patients with β-mercaptolactate-cysteine disulfiduria (2). Studies concerning the biosynthesis of HCETC have been performed using rat tissues, and it has been found that the 3-mercaptolactate portion is formed from L-cysteine through the transamination of L-cysteine and the reduction of the resulting 3-mercaptopyruvate (3, 4).

It has been shown that 3-mercaptopyruvate is a good *in vitro* substrate for lactate dehydrogenase (EC 1.1.1.27) crystallized from heart muscle (5-8). Therefore, lactate dehydrogenase has been considered to catalyze the reduction of 3-mercaptopyruvate *in vivo*, but studies have not been undertaken to confirm the involvement of lactate dehydrogenase in this reaction. Present paper reports that the reduction of 3-mercaptopyruvate catalyzed by rat liver homogenate and by purified rat liver lactate dehydrogenase is inhibited by anti-lactate dehydrogenase antiserum, indicating that 3-mercaptopyruvate reduction in the liver is catalyzed by lactate dehydrogenase.

* To whom correspondence should be addressed.

Materials and Methods

Materials. Ammonium 3-mercaptopyruvate was synthesized according to Kun (6). Sodium pyruvate was obtained from Wako Pure Chemical Ind., Osaka, Japan. Nicotinamide adenine dinucle-

otide, reduced form, (NADH) was purchased from Oriental Yeast Co., Ltd., Tokyo, Japan. Phenylmethylsulfonyl fluoride (PMSF) was from Sigma Chemical Company, St. Louis, MO, USA. Dithiothreitol (DTT) and Combithek, calibration proteins for SDS-gel electrophoresis, were obtained from Boehringer Manheim Biochemicals, Manheim, FRG. Bovine serum albumin, fraction V, was the product of Armour Pharmaceutical Company, Kankakee, IL, USA. Male Wistar rats weighing about 350 g were used for the purification of lactate dehydrogenase. Male Japanese white rabbits weighing 2.5-2.8 kg were used for the preparation of anti-lactate dehydrogenase antiserum. Freund's complete adjuvant was obtained from Difco Laboratories, Detroit, MI, USA.

Purification of rat liver lactate dehydrogenase. Forty grams of rat liver was used, and all steps were carried out at 0-4°C except for ethanol fractionation.

Preparation of rat liver homogenate, ethanol fractionation and ammonium sulfate fractionation. These procedures were performed as previously described (9, 10) except for the use of 3 mmole PMSF per g of rat liver at homogenization. The precipitate obtained by ammonium sulfate fractionation was suspended in 14.5 ml of water, and dialyzed against 20 mM potassium phosphate buffer, pH 6.0 (buffer A). The suspension was centrifuged at 7,500×*g* for 30 min, and the resulting supernatant was separated.

Affinity elution from CM-Sephadex C-50 column. The supernatant (30 ml) obtained above was mixed with 60 ml of buffer A, and the mixture was applied to a column of CM-Sephadex C-50 (4.2 cm, diameter, ×2.6 cm) equilibrated with buffer A. Then, the column was washed with 258 ml of 15 mM potassium phosphate buffer, pH 6.6 (buffer B) containing 0.2 mM EDTA. Elution was performed with 64.5 ml of buffer B containing 0.2 mM NADH. Solid ammonium sulfate (0.5 g/ml) was slowly added to the effluent. After standing for 1 h, centrifugation was performed at 11,000×*g* for 40 min. The precipitate was dissolved in buffer A and dialyzed against 10 mM sodium phosphate buffer, pH 7.4 (buffer C).

Chromatography with DEAE-Sephadex A-50 column. The dialyzed solution (3.8 ml) was applied to a column of DEAE-Sephadex A-50 (1.5 cm, diameter, ×27 cm) equilibrated with buffer C. Elution was performed with the same buffer, collecting 4-ml fractions. The fractions showing a specific activity above 650 U/mg were combined.

Assay of lactate dehydrogenase. Lactate dehydrogenase activity was determined as described elsewhere (11).

Protein determination. The protein concentration was determined by the biuret method (12) and Lowry's method (13) with bovine serum albumin as a standard, and by ultraviolet absorption (10, 12).

Sodium dodecyl sulfate(SDS)-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (14).

Preparation of rabbit antiserum against the purified rat liver lactate dehydrogenase. Rabbit antiserum was prepared according to Carlotti *et al.* (9).

Ouchterlony double diffusion analysis and immunoelectrophoresis. A modification of previously described methods (9) was employed. A 0.9% agar solution in barbital-acetate buffer (pH 8.6, ionic strength 0.1) was used for preparation of agar gel plates (thickness 1 mm). In the immunoelectrophoresis procedure, antigens were electrophoresed for 2 h at 1.5 mA/cm.

Inhibition by the antiserum of the reduction of 3-mercaptopyruvate. The purified rat liver enzyme or rat liver homogenate was incubated with the antiserum at 37°C for 1 h in a total volume of 1.0 ml. Incubation mixtures lacking the antiserum or materials from rat liver, or containing serum from a control rabbit instead of the antiserum were incubated as controls. After standing at 4°C for 12 h, the mixtures were centrifuged at 24,000×*g* for 30 min. Enzyme activities in the resulting supernatants were assayed as follows. The assay mixture (3.0 ml) for pyruvate reduction contained 0.2 mM NADH and 1.6 mM of sodium pyruvate in buffer D, and that for 3-mercaptopyruvate reduction contained 0.2 mM NADH, 16 mM ammonium 3-mercaptopyruvate and 3.3 mM DTT in buffer D. 3-Mercaptopyruvate solution was prepared just before use and kept in an ice bath. The enzyme reaction was started by adding 50 μl of the supernatant and conducted at 30°C while recording the absorbance at 365 nm. The precipitate obtained by the above centrifugation

from the incubation mixture containing rat liver homogenate was suspended in 1.0 ml (final volume) of buffer D (0.1 M potassium phosphate containing 0.1 M ammonium sulfate, pH 7.6) containing 1 mM DTT, and sonicated at 20 kHz for 10 min. The suspension was centrifuged at 24,000×*g* for 30 min, and the resulting supernatant was assayed for enzyme activities as described above.

Polyacrylamide gel electrophoresis. Gel electrophoresis and activity staining for lactate dehydrogenase were carried out as previously described (15) except that 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was used instead of nitroblue tetrazolium.

Results and Discussion

Properties of purified lactate dehydrogenase and its antiserum. Table 1 summarizes the procedure and results of the purification of lactate dehydrogenase from rat liver. The overall yield was 28%, and the final purification was 234-fold.

In the present purification steps, affinity elution chromatography with CM-Sephadex C-50 was very effective, achieving 14-fold purification. As shown in Fig. 1, the purified enzyme was found to be isoenzyme M_4.

Fig. 2 shows SDS-polyacrylamide gel electrophoresis of the purified enzyme. The preparation exhibited a single band which corresponded to a molecular weight of 36,500, indicating that the lactate dehydrogenase preparation obtained by the present purification steps was homogeneous.

Specificity of the antiserum prepared against the purified enzyme was examined by means of Ouchterlony double diffusion analysis and immunoelectrophoresis. As shown in Figs. 3A and 3B, the antiserum formed only one precipitin line with the purified enzyme and also with the sonicated

Table 1 Summary of purification of rat liver lactate dehydrogenase[a]

Steps	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Homogenate	7540	2.88	100	1.00
Ethanol fractionation	2080	8.26	80	2.87
$(NH_4)_2SO_4$ fractionation	735	17.3	59	6.01
Affinity elution from CM-Sephadex	34.6	245	41	85.1
DEAE-Sephadex chromatography	8.9	675	28	234

a: For details, see the text.

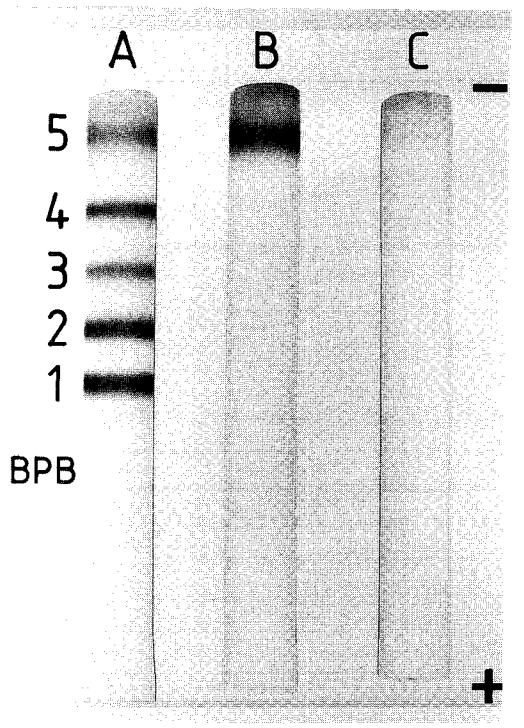

Fig. 1 Polyacrylamide gel electrophoresis of the purified rat liver lactate dehydrogenase. Gel electrophoresis and activity staining for lactate dehydrogenase was performed as described (15). Rat kidney was homogenized with 2 volumes of 0.15 M potassium phosphate containing 0.15 M ammonium sulfate (pH 7.6). One part of the homogenate was diluted to 100 with 40% sucrose. Ten μl of the diluted homogenate was electrophoresed in parallel with 10 μl of the purified lactate dehydrogenase (0.01 mg of protein/ml) in 40% sucrose. Forty % sucrose was used as a control. Gel A, kidney homogenate; gel B, the purified enzyme; gel C, 40% sucrose. Isoenzymes: 1, H_4; 2, H_3M; 3, H_2M_2; 4, HM_3; 5, M_4. BPB, bromophenol blue.

homogenate of rat liver in both analyses, indicating that this antiserum was specific for this lactate dehydrogenase preparation. The fusion of the precipitin line found in Fig. 3A shows that the substances precipitated in both the purified enzyme and the homogenate were immunochemically indistinguishable.

Inhibition of the reduction of pyruvate and 3-mercaptopyruvate by the antiserum against lactate dehydrogenase. Table 2 summarizes the effect of anti-lactate dehydrogenase antiserum on the reduction of pyruvate and of 3-mercaptopyruvate by the purified rat liver lactate dehydrogenase. Neither pyruvate-reducing activity nor 3-mercaptopyruvate-reducing activity was detected in the supernatant from the incubation mixture containing the purified enzyme and the antiserum. This lack of activity indicates that the puri-

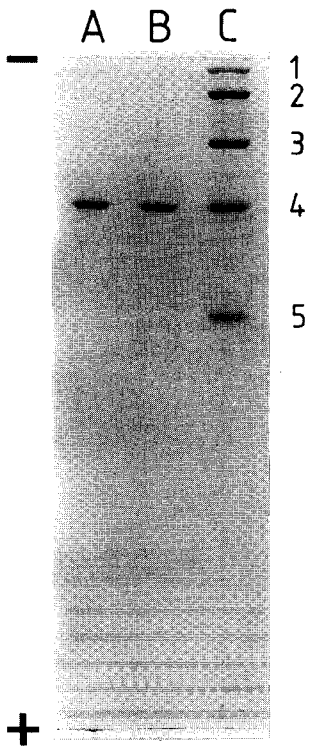

Fig. 2 SDS-polyacrylamide gel electrophoresis of the purified rat liver lactate dehydrogenase. Electrophoresis was carried out according to the method of Laemmli (14) using 15% polyacrylamide slab gel. The purified rat liver lactate dehydrogenase was analyzed after treatment in 62.5 mM Tris chloride, pH 6.8 containing 100 mM DTT and 2% SDS at 25°C for 30 min. Proteins were stained with Coomassie Brilliant Blue R-250. Lanes A and B, 0.9 μg of the purified enzyme. Lane C, molecular weight standards: 1, α_2-macroglobulin, reduced, (Mr, 170,000); 2, phosphorylase b (97,400); 3, glutamate dehydrogenase (55,400); 4, lactate dehydrogenase (36,500); 5, trypsin inhibitor (20,100).

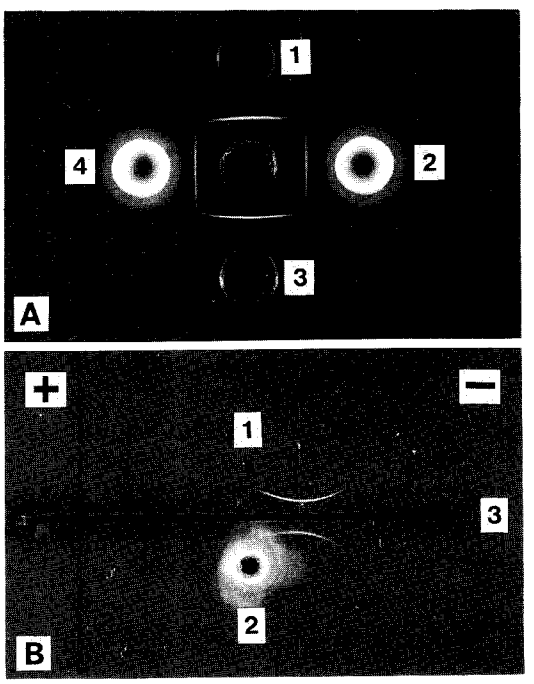

Fig. 3 Ouchterlony double diffusion analysis (A) and immunoelectrophoresis (B). The antiserum against the purified rat liver lactate dehydrogenase was prepared and immunochemical experiments were performed as described under Materials and Methods. Purified lactate dehydrogenase solution in 0.1 M potassium phosphate containing 0.1 M ammonium sulfate (pH 7.6) (buffer D) or 33% rat liver homogenate prepared with buffer D and sonicated at 20 kHz for 10 min was used as antigen. Incubation was conducted for 24 h at 25°C in a humidified incubator. A: 1 and 3, 10 μl of purified rat liver lactate dehydrogenase solution (1 mg of protein/ml); 2 and 4, 10 μl of 33% rat liver homogenate sonicated at 20 kHz for 10 min; center well, 10 μl of 1:2-diluted antiserum prepared against the purified rat liver lactate dehydrogenase. B: 1, 3 μl of purified rat liver lactate dehydrogenase solution (10 mg of protein/ml); 2, 12 μl of 33% rat liver homogenate sonicated at 20 kHz for 10 min; 3, 50 μl of antiserum prepared against the purified rat liver lactate dehydrogenase.

Table 2 Inhibition by anti-lactate dehydrogenase antiserum of reduction of pyruvate and 3-mercaptopyruvate by the purified rat liver lactate dehydrogenase[a]

Incubation with	Remaining activity in the supernatant μmol·ml^{-1}·min^{-1}(%)	
	Substrate	
	3-Mercaptopyruvate	Pyruvate
0.9% NaCl	0.80±0.02(100.0)	3.34±0.03(100.0)
Control serum	0.80±0.02(100.0)	3.28±0.00(98.2)
Anti-LDH antiserum	0.00±0.01(0.0)	0.01±0.06(0.3)

a: Purified rat liver lactate dehydrogenase (4.5 μg protein) was incubated with 0.1 ml of 0.9% NaCl, control serum from a non-immunized rabbit, or anti-lactate dehydrogenase (LDH) antiserum in 1.0 ml (final volume) of incubation mixture containing 10 mg of bovine serum albumin, 1 mM DTT, 0.1 M potassium phosphate (pH 7.6) and 0.1 M ammonium sulfate. After centrifugation, the remaining activities in the supernatants were determined. Values are means±SD obtained from at least 2 separate experiments. Values in parentheses are the percentage of that with 0.9% NaCl. For details, see Materials and Methods.

Table 3 Inhibition by anti-lactate dehydrogenase antiserum of reduction of pyruvate and 3-mercaptopyruvate by rat liver homogenate[a]

Incubation with	Remaining activity in the supernatant μmol·ml^{-1}·min^{-1}(%)	
	Substrate	
	3-Mercaptopyruvate	Pyruvate
0.9% NaCl	3.938±0.009(100.0)	16.57±0.08(100.0)
Control serum	3.993±0.063(101.4)	16.22±0.09(97.9)
Anti-LDH antiserum	0.036±0.013(0.9)	0.062±0.009(0.4)

a: In this experiment, rat liver homogenate was used in place of the purified rat liver lactate dehydrogenase in Table 2. Rat liver was homogenized with 2 volumes of 0.15 M potassium phosphate buffer (pH 7.6) containing 0.15 M ammonium sulfate, using a Potter-Elvehjem type homogenizer equipped with a Teflon pestle. The homogenate (0.1 ml) was incubated with 0.6 ml of 0.9% NaCl, control serum from a non-immunized rabbit, or anti-lactate dehydrogenase (LDH) antiserum in 1.0 ml (final volume) of incubation mixture containing 1 mM DTT, 0.1 M potassium phosphate pH 7.6) and 0.1 M ammonium sulfate. See footnotes to Table 2. For details, see Materials and Methods.

fied enzyme was completely precipitated by the antiserum.

Table 3 is a summary of the inhibition by the antiserum of the reduction of pyruvate and of 3-mercaptopyruvate by the rat liver homogenate. After the homogenate was incubated with the antiserum, the incubation mixture was centrifuged. The resulting supernatant exhibited a very low activity, far less than 1% of the controls in both the reduction of pyruvate and of 3-mercaptopyruvate.

Trace activities of isoenzymes HM_3 and H_2M_2 were detected besides M_4 by activity staining after polyacrylamide gel electrophoresis of the homogenate. Therefore, it was assumed that there was a possibility that the trace values of the activity shown in Table 3 were due to these isoenzymes. However, no lactate-dehydrogenase activity corresponding to HM_3 or H_2M_2 was detected upon activity staining of the supernatant obtained after the incubation of the homogenate with the antiserum. When rat kidney homogenate was incubated with the antiserum against purified rat liver lactate dehydrogenase, no M-subunit-containing isoenzyme was detected as shown in Fig. 4. These results indicate that HM_3 and H_2M_2 were also precipitated by the antiserum.

In order to examine the possibility of the non-specific co-precipitation of a 3-mercaptopyruvate-reducing enzyme besides lactate dehydrogenase, the precipitate formed by the incubation of the homogenate with the antiserum was suspended in buffer D containing 1 mM DTT, and the suspension was sonicated as described under Materials and Methods. The supernatant of the sonicated suspension exhibited negligibly low activity similar to those in Table 3.

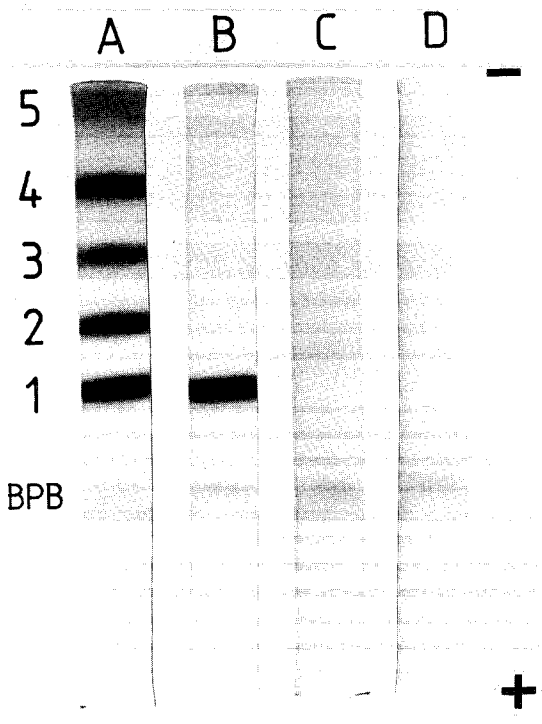

Fig. 4 Polyacrylamide gel electrophoresis of the supernatant obtained after incubation of the rat kidney homogenate with the antiserum against rat liver lactate dehydrogenase. Gel electrophoresis and activity staining were performed as described (15). The incubation mixture (1.0 ml) containing 0.1 ml of the rat kidney homogenate prepared as described in the legend to Fig. 1, 0.6 ml of anti-lactate dehydrogenase antiserum, 1 mM DTT, 0.1 M potassium phosphate (pH 7.6) and 0.1 M ammonium sulfate was incubated at 37°C for 1 h. Incubation mixtures lacking the homogenate or the antiserum were incubated as controls. After standing at 4°C for 24 h, the mixtures were centrifuged at 24,000×*g* for 30 min. Each resulting supernatant was diluted ten-fold with 40% sucrose. Ten μl of each diluted supernatant was electrophoresed. Gel A, incubation mixture lacking the antiserum; gel B, complete incubation mixture; gel C, incubation mixture lacking the homogenate; gel D, incubation mixture lacking both the homogenate and the antiserum. Isoenzymes: 1, H_4; 2, H_3M; 3, H_2M_2; 4, HM_3; 5, M_4. BPB, bromophenol blue.

All results obtained in the present study clearly show that the reduction of 3-mercaptopyruvate in rat liver is catalyzed by lactate dehydrogenase, and not by other enzymes, confirming the previous assumption.

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References

1. Ubuka T, Kobayashi K, Yao K, Kodama H, Fujii K, Hirayama K, Kuwaki T and Mizuhara S: S-(2-Hydroxy-2-carboxyethylthio)cysteine and S-(carboxymethylthio)cysteine in human urine. Biochim Biophys Acta (1968) **158**, 493-495.
2. Crawhall JC: β-Mercaptolactate-cysteine disulfiduria; in The Metabolic Basis of Inherited Disease, Stanbury, Wyngaarden and Fredrickson eds, 4th Ed, McGraw-Hill Book Company, New York (1978) pp 504-513.
3. Ubuka T, Umemura S, Yuasa S, Kinuta M and Watanabe K: Purification and characterization of mitochondrial cysteine aminotransferase from rat liver. Physiol Chem Phys (1978) **10**, 483-500.
4. Ubuka T, Akagi R, Kiguchi S, Taniguchi M and Mikami H: Biosynthesis of S-(2-hydroxy-2-carboxyethylthio)cysteine (3-mercaptolactate-cysteine disulfide) by the rat heart. Biochem Int (1983) **6**, 291-296.
5. Meister A: Enzymatic preparation of α-keto acids. J Biol Chem (1952) **197**, 309-317.
6. Kun E: The reaction of β-mercaptopyruvate with lactic dehydrogenase of heart muscle. Biochim Biophys Acta (1957) **25**, 135-137.
7. Ubuka T, Ishimoto Y and Kasahara K: Determination of 3-mercaptopyruvate-cysteine disulfide, a product of oxidative deamination of L-cysteine by L-amino acid oxidase. Anal Biochem (1975) **67**, 66-73.
8. Cooper AJL, Haber MT and Meister A: On the chemistry and biochemistry of 3-mercaptopyruvic acid, the α-keto acid analog of cysteine. J Biol Chem (1982) **257**, 816-826.
9. Carlotti RJ, Garnett GF, Hsieh WT, Smucker AA, Vestling CS and Morris HP: Comparison of purified lactate dehydrogenases from normal rat liver and Morris hepatomas in rats and in culture. Biochim Biophys Acta (1974) **341**, 357-365.
10. Hsieh WT and Vestling CS: Lactate dehydrogenase from rat liver (E. C. No. 1.1.1.27). Biochem Prep (1966) **11**, 69-75.
11. Vassault A: Lactate dehydrogenase, UV-method with pyruvate and NADH; in Methods in Enzymatic Analysis, Bergmeyer and Grassl eds, 3rd Ed, Vol 3, Verlag Chemie, Weinheim (1983) pp 118-126.
12. Layne E: Spectrophotometric and turbidimetric methods for measuring proteins; in Methods in

Enzymology, Colowick and Kaplan eds, 1st Ed, Vol III, Academic Press, New York (1957) pp 447-454.

13. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ: Protein measurement with the Folin phenol reagent. J Biol Chem (1951) **193**, 265-275.
14. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (1970) **227**, 680-685.
15. Dietz AA and Lubrano T: Separation and quantitation of lactic dehydrogenase isoenzymes by disc electrophoresis. Anal Biochem (1967) **20**, 246-257.

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