Preparation of a Sulfurtransferase Substrate, Sodium 3-Mercaptopyruvate, from 3-Bromopyruvic Acid and Sodium Hydrosulfide

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Sodium 3-mercaptopyruvate (3-MP), which is a sulfur donor substrate for sulfurtransferases, was synthesized directly from 3-bromopyruvic acid in anhydrous methanol at 4 °C, by employing sodium hydrosulfide as a sulfhydryl compound instead of hydrogen sulfide gas. The purity of 3-MP was determined to be 97.9 and 98.6% by assay with *N*-ethylmaleimide and with reduced sodium adenine dinucleotide and lactate dehydrogenase, respectively.

Keywords sodium 3-mercaptopyruvate; synthesis; sodium hydrosulfide; sulfurtransferase substrate; *N*-ethylmaleimide; lactate dehydrogenase

3-Mercaptopyruvate (3-MP) is known to be a sulfur donor substrate for 3-mercaptopyruvate sulfurtransferase (EC 2.8.1.2). 3-MP is believed to be formed metabolically from cysteine by transamination,[1,2] and participates in a transfer ribonucleic acid (tRNA) sulfurtransferase-catalyzed reaction with pyrimidine base in tRNA as the acceptor.[3–5] It is of interest to note that 3-MP is an excellent substrate of lactate dehydrogenase (LDH, EC 1.1.1.27), being reduced to 3-mercaptolactate,[6,7] which is found in the urine of normal subjects in a small quantity as its mixed disulfide with cysteine[8]; a large amount of the disulfide is excreted in the urine of patients with 3-mercaptopyruvate[9,10] or 3-mercaptolactate[11–13] disulfiduria, in which 3-mercaptopyruvate sulfurtransferase is deficient.

It has been reported that 3-MP might contribute to the increase of polyploidy *in vivo*, especially in tumors lacking desulfurase enzymes.[14]

In order to elucidate the physiological function and enzymic mechanism of the sulfurtransferases and the 3-MP pathway of cysteine metabolism, it is necessary to develop a method for assay of the enzyme. Thus, a convenient method for the preparation of sufficiently pure 3-MP is needed, because 3-MP is not commercially available in Japan.

3-MP has been synthesized directly as its ammonium[7] or sodium[15] salt from 3-bromopyruvic acid and either concentrated ammonium hydroxide or anhydrous methanolic sodium hydroxide solution saturated with dry hydrogen sulfide gas. However, these methods are laborious and it is difficult to obtain a pure product; 3-MP easily undergoes aldol condensation under basic conditions to yield the unstable dimer.[16]

In this paper we describe a more convenient preparation of the sodium salt of 3-MP from 3-bromopyruvic acid by using sodium hydrosulfide as a sulfhydryl compound instead of hydrogen sulfide gas.

Experimental

Apparatus Ultraviolet (UV) and infrared (IR) spectra were measured with Hitachi U-2000 and Shimadzu IR-420 spectrometers, respectively.

Materials 3-Bromopyruvic acid was obtained from Kodak Co., Ltd. Reduced sodium adenine dinucleotide (NADH) was purchased from Oriental Yeast Co. LDH from pig heart (400 U/ml) was purchased from Boehringer Co., Ltd. *N*-Ethylmaleimide (NEM) was purchased from Kanto Chemical Co., Inc. All other chemicals and solvents were of the purest grade available.

Purification of Sodium Hydrosulfide Commercial sodium hydrosulfide (20.0 g, about 70% purity) was dried overnight in a sulfuric acid desiccator under reduced pressure. The dried sodium hydrosulfide (15.0 g) was dissolved in ethanol (100 ml, 99.5%) and filtered through a G-4 filter. Then, 1.6 volumes of ether (water content: less than 0.1%) was added to the filtrate, and the mixture was allowed to stand for 1 h, then filtered through a G-4 filter. An equivolume of ether was added again to the filtrate to give a precipitate of sodium hydrosulfide. The resulting precipitate was collected by filtration and dried in a sulfuric acid desiccator *in vacuo*, yielding 6.5 g of pure anhydrous sodium hydrosulfide as a white powder.

Preparation of 3-MP from Sodium Hydrosulfide and 3-Bromopyruvic Acid The purified sodium hydrosulfide (2.0 g) was dissolved in anhydrous methanol (10 ml) and cooled in an ice-bath. 3-Bromopyruvic acid (3.0 g) was also dissolved in anhydrous methanol (10 ml) and cooled in a mixture of salt and ice (4 °C). The cooled sodium hydrosulfide solution was added dropwise to solution of 3-bromopyruvic acid at regular intervals (one drop/10 s) with stirring. The dropping time of sodium hydrosulfide was 1 to 1.5 h. After the sodium hydrosulfide had been added, the reaction mixture was stirred for another 15 min. Addition of ethanol to the mixture gave a precipitate which was collected with a G-4 filter. The precipitate was washed with ethanol (20 ml) to remove a yellow compound and dried in a silica gel desiccator *in vacuo*. The dried white precipitate (3.0 g) was dissolved in 0.01 N HCl (20 ml), and the solution was diluted with 3 volumes of ethanol and allowed to stand for 4 to 5 h in a refrigerator to give 3-MP sodium salt as crystals. The crystals were collected with a G-4 filter and washed with 75% ethanol (50 ml), followed by 99.5% ethanol (200 ml), giving pure sodium 3-MP dihydrate as colorless needles of mp above 300 °C, in a yield of above 60%. *Anal.* Calcd for $C_3H_3NaO_5S \cdot 2H_2O$: C, 20.2; H, 4.0. Found: C, 20.3; H, 4.1. The 2,4-dinitrophenylhydrazone of 3-MP was prepared by the known procedure[17] as reddish orange needles from ethanol, mp 155—158 °C (literature: 161—162 °C[18]). *Anal.* Calcd for $C_9H_8N_4O_6S$: C, 36.00; H, 2.69; N, 18.66. Found: C, 36.20; H, 2.63; N, 18.68. The anhydrate of 3-MP was prepared by drying the dihydrate over P_2O_5 in a vacuum in an Abderhalden pistol at 78 °C until it reached constant weight. For the quantitative determination of 3-MP with NEM and LDH, the dihydrate form was employed.

Determination of Sulfhydryl Group with NEM The content of sulfhydryl group in 3-MP was determined according to the method of Alexander.[19] To 0.8 mM NEM in 3 ml of 0.1 M phosphate buffer (pH 6.0), 0.2 ml of 3.82 mM 3-MP was added and the mixture was allowed to stand for 10 min at room temperature. The content of sulfhydryl group in 3-MP was determined from the difference in absorption at 300 nm between the solution and 0.8 mM NEM in the same buffer, using the molar absorptivity of NEM of 620 $M^{-1} cm^{-1}$.

Assay of 3-MP by NADH and LDH To 3.0 ml of 0.1 M phosphate buffer (pH 7.4) containing 0.15 mM NADH and 2.0 U/ml LDH, 50 μl of 3.06 mM 3-MP was added. The mixture was allowed to stand for 10 min at room temperature, then the decrease in absorbance of NADH at 340 nm was measured. The reaction was completed within 5 min. The amount of 3-MP was calculated by using the molar absorptivity of NADH of 6220 $M^{-1} cm^{-1}$.

Results and Discussion

Preparation of Sodium Salt of 3-MP from Sodium Hydrosulfide and 3-Bromopyruvic Acid The sodium salt of 3-MP was easily obtained by direct sulfhydration of 3-bromopyruvic acid with sodium hydrosulfide in anhydrous methanol at 4 °C. As shown in Table I, when 3-bromopy-

Table I. Sulfhydration of 3-Bromopyruvic Acid (A) with Sodium Hydrosulfide (B)

Method 1[a]				Method 2[b]			
A (%)	B (%)	Molar ratio (A:B)	Yield (%)	A (%)	B (%)	Molar ratio (A:B)	Yield (%)
60	15	1:1	15	60	10	1:2	46
60	15	1:2	31	60	15	1:2	50
60	15	1:3	25	60	20	1:2	56
60	7.5	1:2	26	45	20	1:2	58
60	30	1:5	—[c]	30	20	1:2	63
45	15	1:2	34	15	20	1:2	49
30	15	1:2	38	30	30	1:2	56
15	15	1:2	23				

a) The anhydrous methanol solution of A was added dropwise to the anhydrous methanol solution of B over a period of 60 min at 4 °C. *b*) B was added dropwise to A. Other conditions were the same as those of method 1. *c*) Yield not determined.

Table II. Effects of Reaction Temperature and Dropping Time on the Sulfhydration of 3-Bromopyruvic Acid with Sodium Hydrosulfide

Temperature (°C)	Dropping time (min)	Yield (%)
4	60	59
10	60	19
25	60	—[a]
4	45	58
4	30	51
4	90	42

The anhydrous methanol solution of sodium hydrosulfide (20%) was added dropwise to the anhydrous methanol solution of 3-bromopyruvic acid (30%) in a molar ratio of 2:1. *a*) Yield not determined.

ruvic acid was added dropwise to sodium hydrosulfide according to the conventional method (method 1),[7,15] better yields were obtained with a molar ratio of 1 part of the acid to 2 parts of sodium hydrosulfide. The yield was also affected by the concentrations of the reactants, but did not exceed 40% at any concentration tested.

On the other hand, when sulfhydration was performed by dropwise addition of sodium hydrosulfide to an anhydrous methanol solution of 3-bromopyruvic acid, with the 1:2 molar ratio of the acid to sodium hydrosulfide, high yields were obtained at all reactant concentrations examined (method 2 in Table I). The best yield was obtained when 30% 3-bromopyruvic acid and 20% sodium hydrosulfide were employed. The reaction temperature and the dropping time of sodium hydrosulfide also affected the yield of 3-MP. The best results were attained at 4 °C for 60 min, as shown in Table II. An increase in temperature reduced the yield, and at 25 °C 3-MP could not be obtained in a pure form.

Thus, the present method is very simple but the yield of 3-MP is affected by the molar ratio, the concentration, and the order of addition of the reactants, as well as the reaction temperature. It must also be emphasized that sodium hydrosulfide should be used after complete removal of the water adducts, otherwise the solubility in anhydrous methanol is poor.

Identification of 3-MP The result of elemental analysis indicated that the sodium salt of 3-MP contains 2 mol of water. The product was positive in the nitroprusside and 2,4-dinitrophenylhydrazine tests. The elemental analysis of the 2,4-dinitrophenylhydrazone derivative was consistent with the theoretical values.

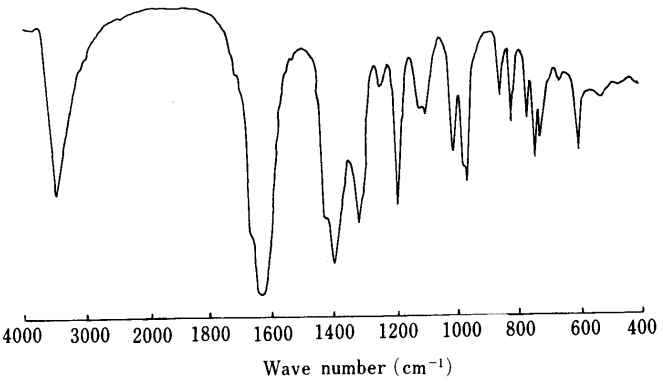

Fig. 1. IR Spectrum of the Anhydrate of Sodium 3-MP in KBr Disk

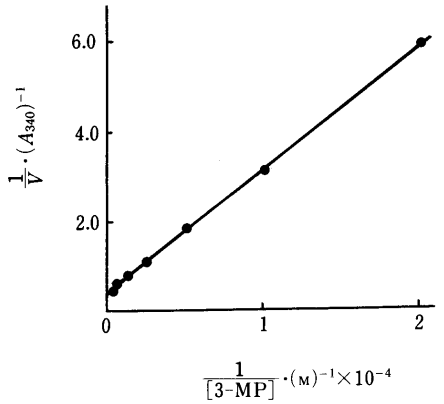

Fig. 2. Double Reciprocal Plot for 3-MP Reduction Catalyzed by LDH

3-MP might be expected to exist as an equilibrium of the keto and the enol forms. Figure 1 shows the IR spectrum of the anhydrate of sodium 3-MP in KBr; the spectrum is similar to that published by Kumler and Kun.[20] From the spectrum, they suggested that 3-MP exists predominantly in the enol form because the absorption band at 3400 cm^{-1} attributed to the enolic hydroxyl group is strong as compared with the absorption of the α-keto group (a small shoulder band at 1680 cm^{-1}). Recently, Cooper *et al.*[16] concluded from isobutane chemical ionization mass and IR spectral data that in the solid and vapor states 3-MP is present as the cyclic dimer, 2,5-dihydroxy-1,4-dithiane-2,5-dicarboxylate, rather than in the enol form; the strong absorption band at 3400 cm^{-1} in Fig. 1 is assigned to the hydroxyl groups of the dimer.

Quantitative Determination of 3-MP with NEM and LDH Since sulfhydryl groups react rapidly with NEM at pH 6.0, their content can be measured from the decrease in absorption of NEM at 300 nm.[19] On the other hand, 3-MP, as well as pyruvic acid, oxidizes NADH in the presence of LDH.[6,7] The initial rate of the reaction is determined by monitoring the decrease in absorbance of NADH at 340 nm.

The purity of 3-MP evaluated by the NEM and the NADH and LDH methods was 97.9% ($n=10$, $CV=3.32\%$) or 98.6% ($n=10$, $CV=2.35\%$), respectively.

The Michaelis constant for 3-MP reduction catalyzed by LDH was found to be 7.8×10^{-4} M at pH 7.4, as shown in Fig. 2. This value is similar to that reported by Kun (8.2×10^{-4})[7] and was 1/15th of the affinity of pyruvate for

LDH (5.2×10^{-5} M).

In conclusion, sulfhydration of 3-bromopyruvic acid to 3-MP was easily performed by employing sodium hydrosulfide as a sulfhydryl compound instead of hydrogen sulfide gas. The present method gave sodium 3-MP in reasonable yield and in adequate purity.

References

1) B. Sörbo, "Metabolic Pathways," Vol. VII, ed. by D. M. Greenberg, Academic Press, New York, 1975, p. 446.
2) A. J. L. Cooper, *Ann. Rev. Biochem.*, **52**, 206 (1983).
3) T.-W. Wong, M. H. Harris, and C. A. Jankowiz, *Biochemistry*, **13**, 2805 (1974).
4) T.-W. Wong, M. H. Harris, and H. P. Morris, *Biochem. Biophys. Res. Commun.*, **65**, 1137 (1975).
5) C. L. Harris, *Nucleic Acid Res.*, **5**, 599 (1978).
6) A. Meister, *J. Biol. Chem.*, **197**, 309 (1952).
7) E. Kun, *Biochim. Biophys. Acta*, **25**, 135 (1957).
8) T. Ubuka, K. Kobayashi, K. Yao, H. Kodama, K. Fujii, K. Hirayama, T. Kuwaki, and S. Mizuhara, *Biochim. Biophys. Acta*, **158**, 493 (1968).
9) U. Hannestad, J. Martensson, R. Sjodahl, and B. Sörbo, *Biochem. Med.*, **26**, 106 (1981).
10) V. E. Shin, M. M. Carney, L. Fitzgerald, and V. Monedjikova, *Pediatr. Res.*, **11**, 464 (1977).
11) C. Crawhall, P. Parker, W. Sneddon, E. P. Young, M. G. Ampola, M. L. Efron, and E. M. Bixby, *Science*, **160**, 419 (1968).
12) A. Niederwieser, P. Giliberti, and K. Baerlocher, *Clin. Chim. Acta*, **43**, 405 (1973).
13) E. A. Law and B. Fowler, *J. Ment. Defic. Res.*, **20**, 99 (1976).
14) J. F. Jackson and K. L. Kiessling, *Science*, **141**, 424 (1963).
15) J. F. Jackson and T. M. Smith, *Biochim. Biophys. Acta*, **121**, 159 (1966).
16) A. J. L. Cooper, M. T. Haber, and A. Meister, *J. Biol. Chem.*, **257**, 816 (1982).
17) D. R. Knapp, "Handbook of Analytical Derivatization Reactions," John Wiley & Son, New York, 1979, p. 340.
18) A. Meister, P. E. Fraser, and S. V. Tice, *J. Biol. Chem.*, **206**, 561 (1954).
19) N. M. Alexander, *Anal. Chem.*, **30**, 1292 (1958).
20) W. D. Kumler and E. Kun, *Biochim. Biophys. Acta*, **27**, 464 (1958).