The Identification of Organic Compounds

II. S-Benzylthiuronium Derivatives of Maleic Acid and Fumaric Acid

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In a previous paper [1] it was shown that adipic acid forms a monosalt and a disalt with S-benzylthiuronium chloride. Maleic acid and fumaric acid have now been included in the work. Details about other dicarboxylic acids will be given in a subsequent paper.

Mono- and disalt of maleic acid. The *monosalt* of maleic acid was first prepared by Donleavy [2] with m.p. 163°C. Veibel and Lillelund [3] found 173—174°C and Chabrier [4] found 186°C. In the standard method proposed by Veibel [5,p.199] *methyl red* is used as an indicator during the neutralisation of the acid. In our hands this technique leads to a mixture probably consisting of the mono- and the disalt (m.p. *ca.* 125—135°C). If, however, at least 3—4 ml of 1 N hydrochloric acid is added to the reaction mixture an almost pure monosalt precipitates. It is highly recommended that the solution of the acid should be half-neutralised (± 5 %), if a pure monosalt is wanted.

Berger [1] has shown that the melting point of an S-benzylthiuronium salt is strongly dependent on the rate of heating. It is now found that the temperature at which the capillary tube is introduced into the bath also is of outmost importance. This, however, is not sufficient to account for the discordant values mentioned above. It has now been found that values ranging from 158°C to 185°C can be recorded by using different methods.

A *disalt* of maleic acid can be obtained by using phenolphthalein as an indicator in the procedure. The formation of the disalt has not previously been observed probably due to a very slow precipitation.

It is known that S-benzylthiuronium chloride itself exhibits polymorphism [3,6]. Morita and Miles [7] have tried to prepare stable polymorphic forms of S-benzylthiuronium salts but they were unsuccessful. However, we are now able to report the first example of polymorphism for an S-benzylthiuronium derivative. The disalt exists in two forms.

Mono- and disalt of fumaric acid. Fumaric acid forms a disalt and its melting point has been recorded as 178°C (Donleavy [2]), as 182—183°C (decomp.) (Veibel and Lillelund [3]) and as 195°C (Vogel [8,p.593]). Veibel and Lillelund [3] state that a monohydrate is formed and that the water of crystallisation cannot be removed by drying at elevated temperature. Donleavy [2] claims an anhydrous salt. We have now been able to prepare three different disalts: An anhydrous salt, a monohydrate and a dihydrate. The temperature during the precipitation is determining the amount of water of crystallisation.

If the salt is used for determination of the equivalent weight by titration with perchloric acid, the amount of water can be determined by a Karl Fischer-titration. It has been found that contrary to the previous findings [3] the anhydrous form can easily be obtained by drying the hydrates. The amount of water of crystallisation has no influence on the melting point. This can be recorded anywhere in the range from 170°C to 210°C, depending on the method used.

An attempt to prepare a pure monosalt of fumaric acid was unsuccessful. The precipitate was analysed as a mixture of the mono- and the disalt.

Stability of hydrates. The stability of di-S-benzylthiuronium fumarate (dihydrate), di-S-benzylthiuronium succinate (dihydrate) and of S-benzylthiuronium acetate (monohydrate) have been determined. The salts were stored in desiccators which maintained the following constant relative humidities: 32 %, 47 %, 58 %, 66 %, and 78 %. Vapour pressure buffers according to Lange [9]. They were all stable during a two months period. Stored over concentrated sulfuric acid the androus forms were obtained after 24 h (fumarate) or after 10 days (acetate and succinate). The fumarate and the succinate can also be dried at 100°C (1 h) whereas the acetate is decomposed at this temperature.

Experimental. The melting points (corrected) were determined in an electrically heated silicone bath [1].

S-Benzylthiuronium hydrogen maleate. Maleic acid (0.3 g) is dissolved in 5 ml of water and 5.0 ml of 0.5 N sodium hydroxide is added. By addition of S-benzylthiuronium chloride (0.5 g dissolved in 5 ml of water) a precipitate is immediately formed. Yield about 90 %; equivalent weight found by titration with 0.1 N perchloric acid [1]: 280.2 (Calc. for $C_{12}H_{14}N_2O_4S$: 282.3). Recrystallisation from 40 % ethanol. The m.p. is highly dependent on the temperature at which the capillary tube is introduced into the silicone bath, and on the rate of heating. Introduced at 145°C: m.p. 158°C (1°/min) and 166°C (4°/min). Introduced at 160°C: m.p. 165°C (1°/min) and 170°C (4°/min). On "Kofler Heizbank" the instantaneous melting point is 183–185°C. It gradually decreases and is about 170°C two minutes later. The melting is always followed by a decomposition.

Di-S-benzylthiuronium maleate. Maleic acid (0.3 g) is dissolved in 5 ml of water and is neutralised with 2 N sodium hydroxide (phenolphthalein). A few drops of 0.5 N hydrochloric acid is added to remove the red colour. S-Benzylthiuronium chloride (1.0 g) is dissolved in 5 ml of water by heating and after cooling the solutions are mixed. No precipitate is formed until after standing for some hours in a refrigerator. Yield about 60 % (Found: Equiv. wt. 223.6; C 53.36; H 5.44. Calc. for $C_{20}H_{24}N_4O_4S_2$: Equiv. wt. 224.3; C 53.55; H 5.39).

The salt exists in two forms: m.p. 122–123°C and 133–134°C. In determining the melting point the capillary tube is introduced into the bath at about 100°C and the rate of heating is 4°/min; a nearly clear melt is obtained at about 123°C and after 2–3 sec the melt solidifies and upon further heating it remelts completely at 133–134°C (decomp.). If the capillary tube is introduced at a temperature between 116°C and 133°C the substance melts immediately and does not solidify again.

It is very difficult to recrystallise the salt. Water and ethanol could not be used as the salt decomposed during the heating. The following procedure has been used: 0.5 g was dissolved in 50 ml acetone by heating on a water bath. A small residue was removed by filtration. 0.3 g pure salt was isolated from the solution after standing several hours in a refrigerator.

Di-S-benzylthiuronium fumarate dihydrate. The temperature during the precipitation of the fumarate must be below *ca.* 40°C in order to obtain a dihydrate. If the temperature is above *ca.* 60°C an anhydrous salt is formed. At about 50°C a salt containing approximately one molecule of water is precipitated. Preparation of the dihydrate: Fumaric acid (0.3 g) is treated as described above (di-S-benzylthiuronium maleate). A precipitate is formed immediately. Yield about 90 % (Found: Equiv. wt. 242.7; H_2O (Karl Fischer-titration) 7.48. Calc. for $C_{20}H_{24}N_4O_4S_2 + 2H_2O$: Equiv. wt. 242.3; H_2O 7.44). Recrystallisation from water taking care that the precipitation takes place below 40°C. Room temperature was preferred in our experiments.

The m.p. can be recorded anywhere in the range from 170°C to 210°C, *e.g.* 179–180°C (decomp.) (4°/min) if the capillary tube is introduced at 170°C. The instantaneous melting point ("Kofler-Heizbank") is about 210°C. It decreases gradually to about 190°C.

Di-S-benzylthiuronium fumarate (anhydrous form). The anhydrous form can be obtained by drying the dihydrate at 100°C for 1 h. It can also be prepared by following the procedure mentioned above for the preparation of the dihydrate except that the two solutions must be 70–80°C when they are mixed. The temperature of the reaction mixture is in this way kept above ca. 60°C during the precipitation. The crystals are isolated immediately after precipitation. Yield about 60 % (Found: Equiv. wt. 226.9. Calc. for $C_{20}H_{24}N_4O_4S_2$: Equiv. wt. 224.3). A Karl Fischer-titration confirmed that it was an anhydrous salt. The melting point was identical with the one found for the dihydrate. It can be recrystallised from water but the temperature during the precipitation has carefully to be kept above *ca.* 60°C.

A transformation of the dihydrate into the anhydrous form and *vice versa* was effected by allowing the precipitation to occur at the temperatures specified above.

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The Enzymic Hydrolysis of S-Phosphorylated Thiols by Bovine Brain

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In a previous communication [1] it was shown that an enzyme hydrolyzing the S—P bond of cysteamine S-phosphate (S-(2-aminoethyl) phosphorothioate) is present in the human erythrocyte. Since a number of S-phosphorylated thiols are now available [2] it was of interest to study the relative rates of enzymic hydrolysis of the S—P bond for a variety of these compounds. Information regarding the effect of substituents on the rate of enzymic hydrolysis could thus be obtained. The effect of substituents on the nonenzymic rate of hydrolysis of the corresponding compounds has been determined earlier [3].

Bovine brain was chosen as an easily accessible source of relatively stable enzyme for these investigations. Brain was chosen as an enzyme source also because of its low blood content. Interference by erythrocyte phosphatases [1] could thus be easily eliminated.

For the preliminary investigations cysteamine S-phosphate was used as a substrate. Pilot experiments with whole brain homogenate showed a pH optimum for hydrolysis of cysteamine S-phosphate around pH 7. The products of hydrolysis were cysteamine and orthophosphate. Mg^{2+} ions were strongly stimulatory. Of various brain fractions tested, the insoluble part of brain homogenate was found to account for 93 % of the total cysteamine S-phosphate hydrolyzing activity present in the homogenate. The insoluble brain fraction was therefore used in all subsequent experiments.

The cysteamine S-phosphate hydrolyzing activity was heat sensitive. 5 min at 50° destroyed 34 % of the initial activity; 5 min at 100° destroyed 84 % and 10 min at 100° destroyed all the initial activity. The hydrolyzing activity thus appeared to be enzymic in nature.

Effect of pH. The effect of pH on the cysteamine S-phosphate hydrolyzing enzyme was determined in the presence of Mg^{2+} ions (5 mM). An optimum was found at pH 7.0. At pH 8.0 the rate of enzymic hydrolysis was 67 % and at pH 6.0 it was 78 % of the rate at pH 7.0.

The enzymic formation of cysteamine from cysteamine S-phosphate was linear with time for about 60 min in a system as described in the legend to Table 2.

Effect of metal ions. Of the metal ions tested Mg^{2+} ions were found to be the most efficient ones in stimulating the cysteamine S-phosphate hydrolyzing activity. The optimum Mg^{2+} ion concentration was 5 mM, which increased the original activity by 210 % at pH 7.0. Of other metal ions tested only Fe^{2+} had an effect comparable to that of Mg^{2+} (Table 1). In contrast to the other cations, nickel ions were found to catalyze the nonenzymic hydrolysis of cysteamine S-phosphate at an extremely high rate (Table 1).

When enzyme was added in the presence of Ni^{2+} ions the amount of cysteamine formed was actually less than in the blank containing no enzyme. A sorbtion of Ni^{2+} ions on the protein probably accounts for this phenomenon.

Table 1. Effect of metal ions on the cysteamine S-phosphate hydrolyzing activity. Test system: cysteamine S-phosphate 5.0 μmoles; metal chloride 5.0 μmoles; enzyme corresponding to 60 mg of fresh brain and tris buffer pH 7.0 90 μmoles in 1.0 ml of water. The enzyme was omitted from the blanks (nonenzymic hydrolysis). Incubation: 30 min at 35°.

Metal ion added	μmoles of cysteamine formed	
	enzymically	nonenzymically
0	0.41	0.00
Mg^{2+}	0.86	0.00
Ca^{2+}	0.30	0.00
Fe^{2+}	0.78	0.04
Co^{2+}	0.06	0.06
Ni^{2+}	—	3.02