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Studies on Polymorphism of Glutathione

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Glutathione is known as an important constituent of all living cells, and has recently been used in clinical fields. Various synthetic routes have been reported by many investigators since Harington and Mead[1] first synthesized the tripeptide in 1935.

The crystal of glutathione is well known as a colorless needle which is easily soluble in water, and its structure has also been discussed.

However, studies on the crystallization conditions of glutathione have scarcely been reported, although some preparative methods are shown.[2-6]

In the present paper, both nuclei formation and the conditions of crystal growth of glutathione were investigated in water and ethanol-water (1 : 2), with varying supersaturated concentration and temperature respectively.

Results

Crystallization in Water System. The glutathione used in this study was repeatedly purified through Cu-salt. Paper electrophoresis did not show any spots of impurities.

Condition of nuclei formation was examined in water without seeding.

The different crystalline nuclei of the glutathione (named type B) were formed when 100% supersaturated solution (100g glutathione/100g water) was maintained above 50°C for 30 min where the crystallization conditions were quite different from those used in the previous reports.

The selective crystal growth of this new type B as well as that of the other one formed under the usual conditions (named type A) may be carried out by seeding the crystalline nuclei of each type to the supersaturated solution under usual crystallization conditions.

In the supersaturated concentrations from 27 to 50%, type A was selectively grown at 10°C after 72 hr, with or without seeding type A. If crystalline nuclei of type B were seeded to the same supersaturated solution at 10°C, only crystal of type B was grown selectively.

In the 100% supersaturated concentration, type

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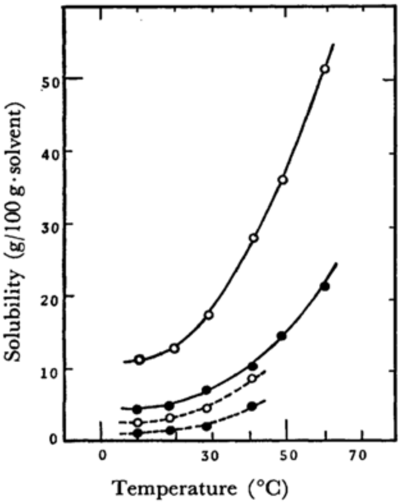

Fig. 1. Solubility curves of type A and B.

— type A
--- type B
○ H_2O
● EtOH-H_2O (2:1)

B was selectively formed with or without seeding type B, when the solution was kept for 30 min at 50°C and then for 72 hr at 30°C. However, if type A was seeded under the same condition, only type A was found in the grown crystal.

The final concentrations in the mother liquor showed 14.0% with A and 5.1% with B respectively after 72 hr at 10°C, and these results suggested that considerable differences of solubilities in water would exist between both types. In fact, these data approximately agreed with those of the solubility curves at 10°C shown below. (Fig. 1)

Crystallization in Ethanol-Water (1 : 2) System. Preparative crystallization of glutathione has been usually carried out by adding suitable amount of ethanol to the aqueous solution. Therefore the crystallization condition was also investigated in ethanol-water (1 : 2) system to check which nuclei of type A or B would be formed.

Remarkable decrease in solubilities of the glutathione (Fig. 1) was found in comparison with those in water system. In this solvent system, only the crystalline nuclei of type A were obtained under conditions in this study.

Chemical and Physicochemical Properties. The new crystalline form named type B was found to be a colorless rigid and fine crystal, while that of type A was a colorless needle.

The solubilities of both types in water were remarkably different as shown in Fig. 1.

Type B was proved to be more stable on storage at 40°C in 100% relative humidity. Thus, the amount remaining unchanged was as much as 75% for B and was only 15% for A after 17 days.

Elemental analysis, paper electrophoresis, $[\alpha]_D$, and mp of type B quite agreed with those of type A.

Both types gave completely the same patterns of NMR (in D_2O, d_6-DMSO, CF_3COOD), ORD (in H_2O) and IR (in D_2O) except IR in KBr tablets. In the pattern of IR spectra, the difference between both types was observed at absorption bands assigned to both amide and carboxyl groups. (Fig. 2)

X-ray diffraction patterns were also measured. It was found that type A showed a very strong peak at d=4.00Å while type B at d=4.19Å, and the spacing of the latter was a little longer than the former.

Discussion

On the Crystallization Conditions. From Fig. 1, the range in which the nuclei of type B were

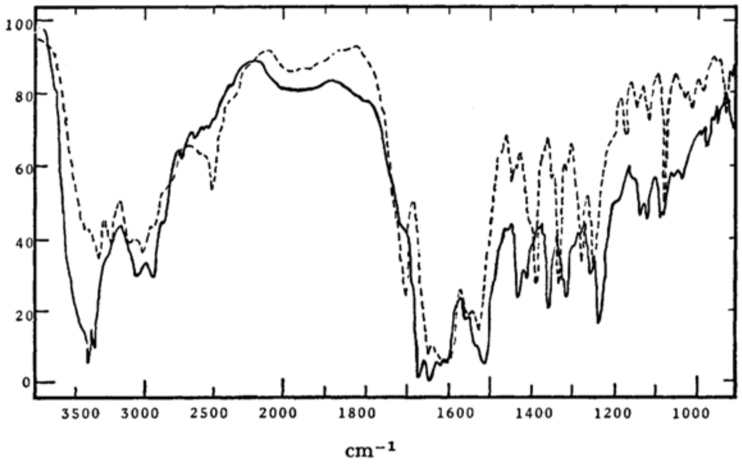

Fig. 2. Infrared spectra in KBr tablets.
---- type A —— type B

formed might exist at higher temperatures or higher concentrations, as the difference between solubility curves of type A and B was much greater. However it was practically difficult to obtain the crystal above 60°C because partial decomposition of glutathione was observed more than an hour later. It is interesting to note that the condition to form nuclei of type B must be restricted within a very small range of temperature and concentration as mentioned above.

It should also be noted that only the selective crystallization of one type took place, with seeding, even in the supersaturated solution where the other type of crystal should be formed without seeding. This fact suggests that the work of nucleus formation of both types would be unusually greater than that of crystal growing. Therefore by seeding one type of nuclei, the same type of crystal would be selectively grown, while nucleus formation of the other type might be disturbed.

Estimation of the Structural Configuration. Many papers about the polymorphism of peptides, in which two different forms are proposed, have been reported. Both of them are "the folded form" with intramolecular hydrogen bonds, and "the extended form" with intermolecular hydrogen bonds. In this connection, IR alternations due to the structural configurations of peptides have also been studied.[7-8] In IR spectra of KBr tablets obtained in this experiment, type B of the glutathione showed a lowering of absorption frequencies at amide ν_{NH} ($\Delta 116\ \mathrm{cm}^{-1}$), amide I ($\Delta 25\ \mathrm{cm}^{-1}$) and amide II ($\Delta 20\ \mathrm{cm}^{-1}$) in comparison with those of type A, respectively. These results would be in satisfactory agreement with those previously reported.

As to the X-ray analysis, Wright[9] reported that the structure of glutathione was held together by a three dimensional network with intramolecular hydrogen bonds. The spacings were calculated as the orthorhombic system, using the unit cell dimensions found by Wright ($a=28.05\pm0.02$Å, $b=8.802\pm0.002$Å, $c=5.630\pm0.002$Å). The calculated value for type A agreed with the observed value in type A.

From these data, the structure of type A must be "the folded form" with intramolecular hydrogen bonds, which is well known previously, and that of the new type B would be "the extended form" with intermolecular hydrogen bonds.

Experimental

Crystallization of Type B. a) Glutathione (10 g) dissolved in water (20 m*l*) was concentrated to total weight 20 g *in vacuo* at 40°C. This supersaturated solution was maintained at 50°C for 30 min with mild stirring, and the solution was allowed to stand for 72 hr at 30°C. Crystal was filtered and washed with a small amount of water and dried in a desiccator under reduced pressure. Colorless, rigid and fine crystal was obtained, yield, 6.5 g. Mp 195°C (dec.), $[\alpha]_{\mathrm{D}}^{20}$ $-17.3°$ (c 2, H_2O). Solubility in water at 10°C, 3.67%. Elemental analysis agreed with the starting glutathione.

b) Nuclei of type B (0.2 g) were seeded to the solution of glutathione (10 g) in water (20 g), and allowed to stand for 72 hr at 10°C. The crystal formed was treated in the same way as above. Yield, 8.9 g. Mp 195°C (dec.), $[\alpha]_{\mathrm{D}}^{20}$ $-17.4°$ (c 2, H_2O). Solubility in water at 10°C, 3.70%.

Apparatus. IR spectra were recorded on a Hitachi IR-G Spectrometer (0.025 mm thickness. KRS-5 sample cell) in D_2O solution and in KBr tablets.

NMR spectra were obtained with an internal standard TMS, JEOL c-60 spectrometer (60 Mc) in D_2O, d_6-DMSO, and CF_3COOD.

X-Ray diffraction patterns were measured with the apparatus of D-3F type made by Rigakudenki, Ltd., in the region of $2\theta=5-40°$ with the use of Cu$K\alpha$ radiation ($\lambda=1.5418$ Å).

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