# GLUCOSULFATASE. IX.
# ON THE SPECIFICITY OF THE ENZYME.

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The name "Glucosulfatase" has been provisionally given to our sulfatase because it seemed to hydrolyse most easily glucose-mono-sulphate. At that time the enzyme-solution had been prepared from *Eulota*, and had only a small activity, consequently the experimental result was somewhat obscure.[1] Meanwhile we have found in *Charonia lampas* a very suitable source of the enzyme[2] and we know now that a very active enzyme can be obtained from its liver by a relatively simple method of purification.[3][4] Glucosulfatase has always been found together with phenosulfatase, but we

(1) This Bulletin, **6** (1931), 258.
(2) This Bulletin, **8** (1933), 65.
(3) *J. Chem. Soc. Japan*, **54** (1933), 377.
(4) This Bulletin, **8** (1933), 148.

have already pointed out the facts which prove that these two enzymes must be different with each other: that the former looses its activity in the alkaline medium while the latter does not,[4] and that each has different tendency toward the adsorption of charcoal.[5] Moreover we have recently succeeded to obtain a solution of glucosulfatase almost free from phenosulfatase.[6] Therefore, it is now quite certain that our enzyme belongs to a new type of sulfatase. We do not know yet, however, whether the name "Glucosulfatase" has been properly adopted or not, for its specificity was tested only with a few substrates at the beginning of this work.[1]

It would be now an urgent problem to settle the specificity of our enzyme and to give it an appropriate name. For this purpose we prepared following aliphatic sulphuric acid esters and examined their hydrolysability by the enzyme: ethyl sulphate, mannite-tetra-sulphate, chondroitinsulphuric acid, glucose-6-mono-sulphate, $\alpha$-methyl-glucoside-mono-sulphate, mono-acetoneglucose-6-mono-sulphate, diacetoneglucose-3-mono-sulphate, fructose-tri-sulphate, galactose-di-sulphate, galactose-tetra-sulphate, mannose-mono- and di-sulphate, sucrose-di-sulphate and the same inverted, maltose-mono-sulphate, starch-di-sulphate. Not many sulphuric acid esters of sugars are known at present and we have had to synthesize some of them for the first time in this laboratory. The chemical constitution of these esters are mostly unknown, so any theoretical deduction from this experiment will be reserved. Fortunately, however, glucose 6-mono-sulphate has been found, as ever, to be the most easily hydrolysable substrate of all. Therefore the name "Glucosulfatase" still seems to be adequate.

The experiments were carried out side by side with those described in the communications V[4] and VII,[5] so that enzyme solution were common to each other, and the experimental procedure was about the same as before. The reaction mixtures had following composition.

| | |
|---|---|
| Substrate solution (with $BaCl_2$) | 25 c.c. |
| Enzyme solution | 25 c.c. |

Chloroform (as an antiseptic) and $BaCO_3$ (as a buffer) were added.

10 c.c. of the above mixture were pipetted out, filtered with charcoal and the clear filtrate was decomposed with HCl; $H_2SO_4$ thus set free was weighed as $BaSO_4$.

Exp. I. The final purification of enzyme was done with $Al(OH)_3$-gel (Communication V. Exp. B. III). Solutions of substrates were about in the same molar concentration as free acids (0.07 Mol.).

(5) *J. Chem. Soc. Japan*, **54** (1933), 1069.
(6) It will be reported in "Glucosulfatase X".

Exp. II. Enzyme solution was further purified with charcoal after the treatment with $Al(OH)_3$-gel (Communication V. Exp. B. IV).

Exp. III. The enzyme solution was prepared according to the direction given in the communication VII, i.e. dil. $NH_4OH$ is added to the crude autolysate from dried liver of *Charonia* and subsequently treated with $Na_2SO_4$ and $BaCl_2$ solution, pH being kept at 9.5–10.0. By such a treatment most of the phosphate which seems to inhibits the action of glucosulfatase, was precipitated and the activity of the enzyme increased. Further treatment was the same as Exp. II.

Results of the experiments are tabulated below.

Exp. I.

| Substrate | $BaSO_4$ mg. | | | % of hydrolysis | |
|---|---|---|---|---|---|
| | Initial | 2 days | 10 days | 2 days | 10 days |
| Glucose-$SO_3$-Ba | 83.2 | 27.6 | 25.4 | 66.8 | 69.5 |
| Galactose-$(SO_3)_2$-Ba | 139.6 | 109.2 | 81.6 | 21.8 | 41.6 |
| Galactose-$(SO_3)_4$-Ba | 291.2 | 224.3 | 211.8 | 23.0 | 27.3 |
| Sucrose-$(SO_3)_2$-Na (inverted) | 87.8 | 57.3 | 52.4 | 34.8 | 40.3 |

Exp. II.

| Substrate | $BaSO_4$ mg. | | % of hydrolysis |
|---|---|---|---|
| | Initial | 2 days | |
| Glucose-$SO_3$-Na | 99.5 | 20.9 | 79.1 |
| α-Methylglucoside-$SO_3$-Ba | 94.6 | 45.9 | 56.5 |
| Sucrose-$(SO_3)_2$-Ba (inverted) | 82.6 | 49.4 | 40.1 |
| Galactose-$(SO_3)_4$-Ba | 78.4 | 58.2 | 38.5 |
| Maltose-$SO_3$-Ba | 93.7 | 61.1 | 34.8 |
| Sucrose-$(SO_3)_2$-Ba | 76.5 | 69.2 (3 days) | 9.5 |
| Diacetoneglucose-$SO_3$-Ba | 108.5 | 100.2 | 7.6 |
| Mannite-$(SO_3)_4$-Ba | 100.1 | 98.7 | 1.4 |
| Chondroitinsulphuric acid (Na) | 121.0 | 120.6 | 0.3 |
| Monoacetoneglucose-$SO_3$-Ba | 80.1 | 80.0 | 0.0 |
| Fructose-$(SO_3)_3$-K | 96.5 | 96.5 | 0.0 |
| Ethyl-$SO_3$-Ba | 149 3 | 149.5 | 0.0 |

Exp. III.

| Substrate | $BaSO_4$ mg. | | % of hydrolysis |
|---|---|---|---|
| | Initial | 2 days | |
| Glucose-$SO_3$-Ba | 113.4 | 9.1 (1 day) | 92.0 |
| Mannose-$SO_3$-Ba | 200.8 | 60.9 | 69.7 |
| Sucrose-$(SO_3)_2$-Ba | 108.3 | 91.8 | 15.2 |
| Starch-$(SO_3)_2$-K [7] | (221.5) | — | 1.0 |

The preparation of substrates.

(1) Glucose-6-mono-sulphate: Soda, this Bulletin, 8 (1933), 37.

(2) Galactose-tetra-sulphate: Akamatsu, *Biochem. Z.*, **142** (1923), 181.

(3) Diacetone-3-mono-sulphate: Ohle, *Biochem. Z.*, **136** (1923), 428.

(4) Monoacetone-6-mono-sulphate: ibid.

(5) Chondroitinsulphuric acid: Jorpes, *Biochem. Z.*, **204** (1929), 354. (prepared from cartilage).

(6) Starch-di-sulphate: Tamba, *Biochem. Z.*, **141** (1923), 274.

(7) Mannite-tetra-sulphate: Favre, *Ann. chim. phys.* (3) **11** (1844), 77. Favre had obtained di-ester, but in my case the product was found to be tetra-ester notwithstanding the same direction was followed. 3.6 Gr. of Ba-salt was obtained from 21 gr. mannite; Ba-content: 34.8%, calculated for $C_6H_8O_6(SO_3)_4Ba_2$: 35.25%.

(8) Sucrose-di-sulphate: Soda, this Bulletin, 9 (1934), 1.

(9) Sucrose-di-sulphate (inverted); The solution of the free acid of (7) was kept for few days at room temperature and then neutralized with $BaCO_3$. By such a treatment the disaccharide is splitted into a mixture of hexose-sulphate.

(10) $\alpha$-Methylglucoside-mono-sulphate: The same direction as (1) was applied to $\alpha$-methylglucoside, but without fermentation with yeast. Yield: 8 gr. as Ba-salt from 20 gr. of glucoside. Content of Ba: 18.45%, calculated from $(C_6H_{10}O_6CH_3SO_3)_2Ba \cdot C_2H_5OH$: 18.7%.

(11) Galactose-di-sulphate: Galactose was, as glucose, treated in pyridin with chlorosulphonic acid. Its crude Ba-salt was purified by crystallizing it as brucine-salt, because the residual galactose could not be fermented off by the ordinary baker's yeast. From 15 gr. galactose 16 gr.

(7) As this substrate is precipitated by Ba-ion, the hydrolysed $H_2SO_4$ was estimated by alkalimetry (instead of $BaCO_3$ Na-acetate was used as a buffer pH: 5.4).

crude Ba-salt, and from the latter 6 gr. crystalline brucine-salt were obtained. Sulphur content of brucine salt: 5.65%; calculated from $C_6H_{10}O_6$ ($SO_3$-brucine)$_2$: 5.67%. $[\alpha]_{5461} = -8.3° \rightarrow -10.1°$ (c = 2.11 in 10% alcohol). Ba-salt used for the experiment was prepared from this brucine salt. Galactose-meno-sulphate could not be obtained.

(12) Maltose-mono-sulphate: 20 gr. maltose dissolved in pyridin was sulphonated with chlorosulphonic acid and by exactly the same treatment as in the case of glucose its Ba-salt was prepared. Yield: 7 gr. The content of Ba: 12.5%; calculated from $(C_{12}H_{21}O_{11}SO_3)_2Ba \cdot C_2H_5OH$: 12.8%. $[\alpha]_{5461} = +97.0°$ (c = 3.36). Its reducing power was increased about 1.44 times by heating its acidic solution at 100°C. for 4 hours (estimated by Hagedorn-Jensen-Hanes' method).

(13) Mannose-mono- and di-sulphate: The method of preparation was exactly the same as glucose-mono-sulphate. Ba-content of its Ba-salt was found to be 22.6% while the calculated value from $(C_6H_{11}O_6SO_3)_2Ba$ is 20.97%. Yield: 20 gr. 10 Gr. of the Ba-salt was changed into brucine-salt by usual way. Relatively insoluble salt of di-ester first crystallized out of its water-solution (about 1.6 gr.), and mono-ester, in the mother-liquor, crystallized out (15 gr.) when acetone was added in the hot solution. Analysis: Brucine-mannose-mono-ester $C_6H_{11}O_6SO_3H \cdot C_{23}H_{26}N_2O_4 \cdot H_2O$, found: 4.80% S; calculated 4.82%. $[\alpha]_{5461} = -14.26°$ (c = 1.825). Brucine-mannose-di-sulphate $C_6H_{10}O_6(SO_3H \cdot C_{23}H_{26}N_2O_4)_2$, found: 5.65% S; calculated: 5.67%.

(14) Fructose-tri-sulphate: Dried fructose (18 gr.) was dissolved in pyridin (20 c.c.) and mixed with an alcoholic solution of pyridin-sulphonic acid (20 gr. in 50 c.c.). Then the mixture was evaporated to syrup at 60–70°C. under reduced pressure and kept at 35°C. for 2 days. Again it was evaporated under the same condition until no more bubbles could be seen; then it was dissolved in cold water and treated with barium hydroxide until it became just a little alkaline. The excess of $Ba(OH)_2$ was precipitated with $CO_2$ and the solution was evaporated at 40°C. under reduced pressure until free from pyridin. A little excess of $H_2SO_4$ was added to it and by treating with PbO and subsequently by passing $H_2S$ the free acid of the acid-ester was obtained as its aqueous solution. It was neutralized with KOH and fermented over night with yeast to remove free sugar. It was filtered, concentrated under reduced pressure and precipitated by adding about its 10 volumes of absolute alcohol. Yield was about 10 gr. According to the analysis of this K-salt the percentage of K was found to be 21.95% and 21.86%, and that of S 17.67% and 17.93%; whereas percentages calculated from the formula $C_6H_9O_6(SO_3K)_3$ are found to be K = 21.95% and S = 17.99%. $[\alpha]_{5461} = -5.4°$ (c = 3.03).

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