AN ENZYME WHICH HXDROLYSES GLUCOSE-MONO-SULPHATE[1] : GLUCO-SULFATASE.

(Preliminary Communication).

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Many studies on the enzymic hydrolysis of sulphuric acid esters were carried out especially by Neuberg and his co-workers. They have already discovered pheno-sulfatase[2] and recently chondro-sulfatase.[3] Another sulfatase is in myrosin. It splits sulphuric acid esters of the glucosides contained in mustard, but it is different from above sulfatases according to Neuberg and is called by him Senfölglukosido-sulfatase.

Glucose-mono-sulphate, synthetically prepared, was not fermented by yeast[4] and also Takadiastase which contains pheno-sulfatase has no power of hydrolysing it.

Yamazaki, in our laboratory, has examined the action of myrosin from mustard upon this compound, but unmistakable result was not obtained though after several weeks very faint hydrolysis was observed (unpublished). It was also noticed that when the solution of the barium salt of this ester was stored long while, some bacteria grew in it and at the same time the deposition of the barium sulphate was remarkable, but we could

(1) Neuberg and Liebermann: *Biochem. Z.*, **121** (1921), 326; Ohle: ibid., **131** (1922), 601, **136** (1923), 428; Soda: ibid., **135** (1923), 621.

(2) Neuberg and Kurono: *Biochem. Z.*, **140** (1923), 295; Neuberg and Linhardt: ibid., **142** (1923), 191; Noguchi: ibid., **143** (1924), 186, **144** (1924), 138; Neuberg and Simon: ibid., **156** (1925), 365; Rosenfeld: ibid., **157** (1925), 434; Neuberg and Wagner: ibid., **161** (1926), 492, **174** (1926), 457; Weinmann: ibid., **205** (1929), 214; Fromgageot: ibid., **208** (1929), 482; Nakamura: ibid., **175** (1926), 226.

(3) Neuberg and Hofmann: *Biochem. Z.*, **234** (1931), 345; Naturw., Heft 23/25 (1931), 484.

(4) Soda: *Biochem. Z.*, **135** (1923), 623.

not decide whether this was due to the bacterial action or merely natural decomposition.

Hirudo medicinalis when fed on the dilute solution of sodium salt of this ester, some increase of the sulphate ion was observed in a few days, yet we have not succeeded to prepare the enzyme solution from it.

At last we have experienced very strong hydrolysing power of snails. Those we collected were of Eulota spp.: *Eulota luhuana*, *Eulota quaesita* and *Eulota pelionphala*. They are commonly found in Tokyo districts, especially in rainy season. From these the enzyme solution was prepared by such a procedure as described in the experimental part. The enzyme solution was brought together with the substrate solution in a stoppered bottle and put aside at room temperature (22°—28°). Every day a portion of this mixture was drawn out and the sulphate ion was estimated after Dennis's nephelometric method of blood sulphate estimation.[1] To compare the turbidity we used a control instead of the standard sulphate solution which is used in the usual procedure. The control was made from a solution of the same composition as the reaction mixture, freshly prepared just before every estimation, applying the same technique as for the reaction mixture. In this way the effect of a trace of sulphate ion which could not be removed from the original solutions was cancelled, and thus the difference of the nephelometric readings corresponds the degree of the enzymic hydrolysis.

The series of data thus obtained are somewhat irregular but on the whole we can clearly recognize the progress of the hydrolysis. In good conditions about 10% of the applied glucose-mono-sulphate was found to be hydrolysed in the course of a week (for these estimations Fiske's benzidine titration method[2] was used).

The action of our enzyme upon the potassium salt of following esters was also examined in order to know its specificity: Ethyl-sulphate, phenol-sulphate, galactose-tetra-sulphate[3] and sucrose-sulphate.[4]

Ethyl-sulphate was not attacked throughout all the experiments, while it gave always positive results with glucose-mono-sulphate. Results with galactose-sulphate and sucrose-sulphate were sometimes negative and sometimes positive, but not very marked.

While phenol-sulphate was very strongly hydrolysed by Enzyme-B, it was only slightly decomposed by Enzyme-A and -D which were found to be very active toward glucose-mono-sulphate. For the preparation of Enzyme-A and -D we applied about the same procedure, but Enzyme-B was

(1) Dennis: *J. Biol. Chem.*, **49** (1921), 311.
(2) Fiske: ibid., **47** (1921), 26.
(3) Akamatsu: *Biochem. Z.*, **142** (1923), 181.
(4) Soda: ibid., **135** (1923), 621.

prepared by somewhat different way as is shown in experimental part. Thus it seems possible to differentiate the glucose-sulphate hydrolysing action of our enzyme from that of pheno-sulfatase; therefore it has, so far as we examined, specific action of hydrolysing glucose-mono-sulphate, and we wish to suggest for this new sulfatase a provisional name "Gluco-sulfatase." Its optimum pH seems to be about 7. We do not know yet whether this enzyme, as Neuberg's chondro-sulfatase, has the faculty of hydrolysing chondroitin-sulphuric acid. An experiment to decide this question is now going on.* Moreover it should also be investigated whether both the pheno-sulfatase action and gluco-sulfatase action of our enzyme due to the one and same enzyme or there exist two different and separable enzymes.

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Experimental Part.

Preparation of enzyme solution. Snails were collected in Tokyo or its suburb. The first collection in which *Eulota pelionphala* predominated was treated as following: Snails were hungered for about a week in a cage, then their shells were removed (50 gr.) and crushed by freezing them in liquid air. Now 100 c.c. of water together with some coarse silica sand, kieselguhr and barium carbonate were added and ground in a mortar throughly by adding chloroform. After four days' autolysis, the enzyme solution was separated by means of the centrifugal machine. We labelled this preparation Enzyme-A.

Enzyme-B was prepared from the second collection, The most part of this collection were *Eulota luhuana*, and weighed 80 gr. when their shells removed. They were treated almost in the same manner as above described, by adding 160 c.c. of water, 15 gr. of kieselguhr and some silica sand, but in this case the addition of barium carbonate was omitted and toluene was used instead of chloroform.

The third collection (the most part *Eulota quaesita*): Snails were hungered for about two weeks, and they have lost much of their activity. They weighed 180 gr. in naked state and were ground in a mortar immediately, without freezing, to puree with some coarse silica sand and chloroform. This puree was kept at room temperature for about a week in a stoppered bottle.

* While this paper was under press, we have ascertained that the gluco-sulfatase did not hydrolyse chondroitin-sulphuric acid which was prepared from tracheal cartilage.

Enzyme-C was prepared from 60 gr. of this puree merely extracting with 120 c.c. of water. After some ten days, to another 60 gr. of the puree 10 gr. of kieselguhr, 10 gr. of barium carbonate and some water were added, ground in a mortar and subsequently by means of centrifugal machine the solution of Enzyme-D was separated.

Manipulations for the estimation of the increase of sulphate ion. For this estimation Dennis's method[1] was applied in the following manner: 2 c.c. of the reaction mixture (composition of which are given at each protocol) were drawn out every day. To this 5 c.c. of $HgCl_2$-HCl mixture were added and filtered through after half an hour with a small amount of animal charcoal (free from sulphate). Of this clear protein free solution 5 c.c. was taken out and then by adding 2.5 c.c. of ammonium nitrate solution and 2.5 c.c. of barium chloride solution the suspension of barium sulphate was formed. Turbidity thus produced was compared with that of the control by means of nephelometer. The control was made, by applying the same manipulation as above stated, from a solution of the same composition as the reaction mixture. This solution was made every time just before use by mixing the substrate solution and the enzyme solution which were kept at the same temperature as the reaction mixture.

I. Experiments with Enzyme-A.

Protocol No. 1. (June 25th, 23°C). The composition of the reaction mixture:

Enzyme solution	Water	Substrate solution
5 c.c.	5 c.c.	10 cc.

The approximate concentration of each substrate solution:

(1)	Sodium glucose-mono-sulphate	(Gl.)	2 %
(2)	Potassium phenol-sulphate	(Phen.)	5 %
(3)	Potassium galactose-tetra-sulphate	(Gal.)	5 %
(4)	Potassium sucrose-sulphate	(Suc.)	5 %
(5)	Potassium ethyl-sulphate	(Ethyl.)	7 %

Days	Gl.	Phen.	Gal.	Suc.	Ethyl.
1	9.2	7.8	10.0	6.1	9.7
2	8.9	8.9	6.7	9.8	10.4
4	4.5*	7.9	8.3	8.1	11.9
5	5.1*	7.3	7.8	8.9	11.9

(1) loc. cit.

* For these figures the control was set at 20.0 mm., as the turbidity was too high.

These figures are nephelometric readings in mm., those of controls being always set at 10.0 mm. This is understood in the following protocols too.

At the sixth day the decomposed amount of sulphate of glucose-mono-sulphate was estimated: 5 c.c. of the reaction mixture was treated with 2 c.c. of trichloroacetic acid (20%) and subsequently filtered through with animal charcoal (free from sulphate). With 5 c.c. of this protein-free filtrate the amount of sulphate was determined by Fiske's benzidine titration method.[1] 1.26 c.c. of NaOH solution (1 c.c. corresponds 0.311 mg. of sulphur) were used. From this value the amounts of sulphate hydrolysed in 1 c.c. of the original reaction mixture is calculated as follows:

$$0.311 \times 1.26 \times 7/5 \times 1/5 = 0.11 \text{ mg. as sulphur.}$$

After the acid hydrolysis of the protein-free filtrate the amount of sulphur was again determined in the same way. 1.14 Mg. of sulphur was found in 1 c.c. of the original reaction mixture. Therefore 0.11/1.14=9.7% of glucose-mono-sulphate ought to be hydrolysed by the enzyme. The influence of sulphate in the control was here neglected because of its small amount.

Protocol No. 2. (July 1st, 25°C.) The composition of the reaction mixture:

Enzyme solution	Buffer solution	Glucose-mono-sulphate (2%)
5 c.c.	5 c.c.	10 c.c

The buffer solution: 1/20 Mol. sodium acetate-acetic acid mixture, except for pH = 8 which was composed of 1/10 mol. ammonium chloride-ammonia.

	pH				
Days	4	5	6	7	8
1	10.6	8.8	8.2	7.6	7.2
2	11.4	10.6	9.4	7.3	8.1
3	9.5	8.9	7.4	6.4	8.4
5	7.4	7.5	6.8	5.0	6.5
6	8.3	7.6	6.7	5.5	7.4

At the seventh day the hydrolysed amount of sulphate was estimated, as in protocol No. 1, with the solution of pH=7. It was found that 12.5% of the total sulphur was titratable as free sulphate ion.

(1) loc. cit.

Protocol No. 3. (July 24th, 24°C.) The composition of the reaction mixture:

	Enzyme solution 10 c.c.	Substrate solution 10 c.c.	
Days	Gl. (6 %)	Phen.	Gal.
1	9.3	10.9	11.5
3	8.0	9.0	10.0
4	6.4	8.7	9.2
5	6.8	8.7	8.6
7	5.1	8.8	8.8

II. Experiments with Enzyme-B.

Protocol No. 4. (July 14th). This experiment was carried out in the thermostat at 35°C., the composition of the reaction mixture being the same as in protocol No. 2. Its pH was kept at 7.0.

Days	1	2	3	4	5
	6.8	3.3	4.5	7.4	6.6

As was expected the enzymic hydrolysis went on very rapidly for the first few days, but later on the nephelometric readings gradually increased as is seen in above data. This singularity is probably due to the natural decomposition of solutions from which the control was prepared owing to the high temperature. Because, both the turbidities increased steadily if we compared each of them separately with that of 1/2000 mol. potassium sulphate.

Protocol No. 5. (July 17th, 26°C.) The composition of the reaction mixtures was the same as that of Protocol No. 1.

Days	Gl. (6 %)	Phen.	Gal.	Suc.	Ethyl.
1	9.8	7.9	10.7	12.3	11.1
3	6.6	6.2	12.5	7.5	13.7

Protocol No. 6. (July 28th, 26°C.) The composition of the reaction mixtures was the same as that of Protocol No. 3.

Days	Gl. (3.8 %)	Phen.
1	8.9	10.3
3	8.7	10.2
4	7.7	4.7
6	7.2	4.5

III. Experiments with Enzyme-C and -D.

Protocol No. 7. (August 4th, 28°C.) The composition of the reaction mixtures was the same as that of Protocol No. 3.

	Enzyme-C		Enzyme-D	
Days	Gl.	Phen.	Gl.	Phen.
1	6.9	10.3	6.4	9.5
2	7.0	9.7	6.6	6.4
4	7.3	10.1	3.8	8.5

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