GLUCO-SULFATASE VI.[1] ON NATURAL SUBSTRATES OF THE ENZYME.

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Received June 14th, 1933. Published July 28th, 1933.

In the preceding papers we have chiefly dealt with the properties, the purification of the enzyme and its distribution in molluscan phylum as well as in organs of the animal. It was, however, since the beginning of our research a problem what rôle does the enzyme, glucosulfatase, play in the physiology of those animals.

It is well known that the substrates of pheno-sulfatase naturally occur in the animal body. Couldn't we expect the corresponding substrate of glucosulfatase? Glucose-mono-sulphate, so far as we know, has not yet been found in nature. We have, however, observed during our experiments that the tissues of those animals seem to contain some organic sulphate and to liberate inorganic sulphate by the enzyme. Therefore,

(1) Gluco-sulfatase. Part I; III; V; this Bulletin, **6** (1931), 258; **8** (1933), 65; **8** (1933), 148. Part II; IV; *J. Chem. Soc. Japan*, **54** (1933), 59, 377.

it would be interesting and worth while to look for such a natural substrate in the body of those animals.

Of course, the merit of the enzyme would not be restricted merely to a single physiological function. It is known that there are some organic sulphates in fronds and in animal tissues, and these might be eaten and digested by those animals which contain gluco-sulfatase. Some kinds of gastropoda, such as *Dolium galea* or *Natica ampla*, are said to excrete sulphuric acid. It is very attractive to suppose some relation between those phenomena and the physiological rôle of our enzyme, but at present, it is merely a speculation. In the present paper, as a preliminary research, we have dealt with the distribution of ethereal sulphate in some forms of gastropoda.

The estimations of organic and inorganic sulphates were made on 5 kinds of animals. On *Viviparus japonicus* Martens (mud-snail) the body parts were roughly separated and the sulphates were estimated in each part. In muscular and organ parts the contents of organic sulphate were nearly equal; in embryo the content was markedly high, being 4-5 times of the content of muscular part. (Table I).

On other 4 species of marine origin the sulphates were determined not separately in parts, but the shell contents were boiled and crushed altogether in each case and their sulphates were determined. In all cases, the contents of organic sulphate (as S) were higher than that of *Viviparus* (9.8 mg.), ranging diversely from 10.4 up to 259 mg. per 100 gr. (see Table II). From the results it would be obvious that the order of activities of enzyme is about the same as that of contents of organic sulphate. The latter, it is worthy of special mention, is just the same as that of the relative weight of the shells, thus suggesting the intimate relation between shell-formation and organic sulphate as well as gluco-sulfatase (see Table III).

The extraction by sodium chloride solution was ineffective in all cases. At the best result (*Viviparus*, muscular part) 1/2.4 of organic sulphate was extracted and at the worst one only 1/6.5 was extracted, and yet it is probable that the organic sulphate in the extract may be due to the suspending proteinous matter. Contrary to chondroitin-sulphuric acid ester, therefore, the substance seems to be difficultly soluble in sodium chloride solution. Alcohol and acetone were much more inefficient for extraction.

Some insoluble parts containing organic sulphate were subjected to peptic digestions, but in many cases they went very slowly. But the embryo of *Viviparus* was dissolved by pepsin-HCl in 2 days and the solution showed the presence of a considerable amount of organic sulphate.

The mucus secreted from fresh *Charonia* by pouring chloroform into its shell showed very interesting properties; it is coagulated by acetone or by boiling, giving gelatinous coagulum and it contains much organic sulphate together with a large quantity of inorganic sulphate. The coagulum did not dissolve in peptic and tryptic mixtures and thus we could not obtain organic sulphate in soluble form. We found, however, the existence of soluble organic sulphate in the filtrate of heat-coagulum. This filtrate, when mixed with the enzyme obtained from the liver of *Charonia*, showed some increase of inorganic sulphate, and thus we could ascertain the hydrolysis of this soluble organic sulphate by gluco-sulfatase. Furthermore, it is clear from our experiment that the insoluble organic sulphate was also hydrolysed by the sulfatase. (58.2% of S of insoluble organic sulphate was hydrolysed during 2 weeks' digestion by the enzyme). From the above results we are convinced of the existence of substrate of gluco-sulfatase probably both in soluble and insoluble form in the bodies of some shell-fishes.

Experimental Details.

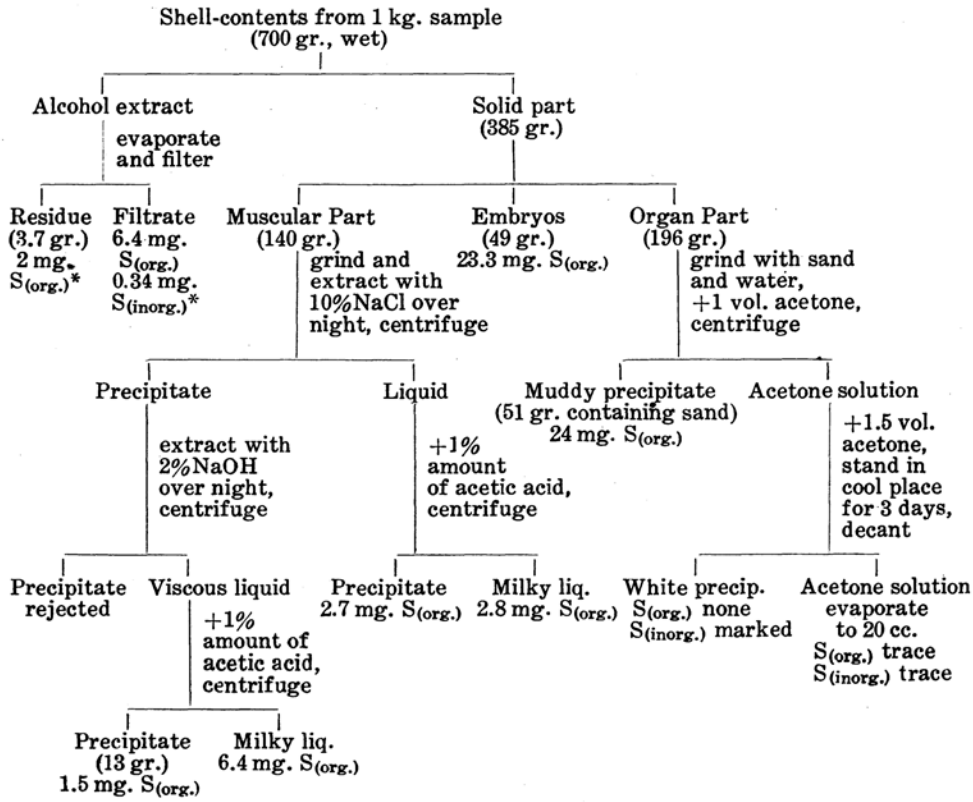
Organic and Inorganic Sulphates in *Viviparus japonicus*, Martens. Sample: Collected in Tokyo at the beginning of July, 1932. 1 kg. of large animals was taken. After washing with running water over night, they were dipped into boiling water for 2 minutes and the shell contents were drawn out, on which the sulphates were determined separately as shown in the following scheme. On liquid parts (extracts) both organic and inorganic sulphates were determined, but on the insoluble parts the organic sulphate only was determined, as there might be no insoluble inorganic sulphate remained in the extract residue. Hydrolysis of organic sulphate was conducted by boiling 50 c.c. of extract (or the suspension of extract residue) with 14 c.c. of conc. HCl for 8-12 hrs. under a reflux condenser.

Table I.

Distribution of organic sulphate in some parts of a body of *Viviparus*.

Parts	S mg.	S mg. per 100 gr. sample
Alcohol extract	8.1	—
Muscular part	13.4	9.6
Embryos	23.3	47.6
Organ part	24	12.2
Total	68.8	(mean) 9.8

Scheme of analysis of *Viviparus japonicus*, Martens.



* $S_{(org.)}$ or $S_{(inorg.)}$ means sulphur of organic or inorganic sulphate.

Analyses of 4 Marine Shell-fishes.

Samples: *Haliotis gigantea* Gmelin (Awabi) } from market.
Turbo cornutus Solander (Sazae) }
Charonia lampas Linne (Bōshu-bora) } from Misaki Marine Biological Station.
Strombus luhuanus Linne (Magaki-gai) }

Their shell-contents were boiled for few minutes, ground to paste with some water and sand (free from sulphate) and extracted by 5-12 times of 10% sodium chloride solution in an ice-chamber for 2 days. The liquids were then centrifuged and the sulphates were determined on the supernatant liquid and the precipitate respectively.

Table II.

	Haliotis	*Turbo*	*Charonia*	*Strombus*
Weight gr.	200	131	167	88 (3 shells)
Shell-content gr. (wet)	134	39	37	24
$S_{(org.)}$ mg. in precipitate	8.5	24.0	78.4	29.2
$S_{(org.)}$ mg. in extract	5.4	13.0	17.3	5.3
$S_{(inorg.)}$ mg. in extract	27.4	17.4	27.0	6.7
Per 100 gr. Shell-content $S_{(org.)}$ mg.	10.4	95	259	144
Per 100 gr. Shell-content $S_{(inorg.)}$ mg.	20.5	44.6	73.0	27.9

In comparing these results with the activities of enzyme reported in the previous paper,[1] we see the order of magnitudes of percentage of organic sulphate is in general same as that of activities of enzyme:

Table III.

	Hydrolysing power of enzyme (%)	Org. Sulphate (mg. S)	Total sulphate (mg. S)	Relative weight of shell $\frac{\text{weight of shell}}{\text{Total weight}} \times 100$
		Per 100 gr. shell-content		
Charonia	74.6	259	332	78
Strombus	18.3	144	171.9	73
Haliotis	12.5	10.4	30.9	33
Turbo	7.2	95	139.6	70

Digestion of the Embryos of *Viviparus* by Pepsin. Some embryos of *Viviparus*, after 1 hour's boiling with water, were digested with pancreatin and pepsin-HCl solutions. After 2 days the embryos disappeared in pepsin mixture, giving white turbid solution, which after clarification by Esbach's picric acid reagent and "Silikatzuckerkohle" showed a considerable amount of organic sulphate.

Mucus secreted from *Charonia*. Fresh *Charonia*, when treated with chloroform, secretes a large amount of mucus. The gelatinous mass, formed by the precipitation of mucus with acetone and subsequent washing with water until free from sulphate, gave on hydrolysis a marked amount of sulphate with a smaller amount of phosphate. On the other hand, soluble organic sulphate was found in the mucus, when it was heated to 100° and the heat-coagulum was filtered off. This soluble organic sulphate was precipitated incompletely by lead acetate, but completely by basic lead acetate.

Hydrolysis of Soluble and Insoluble Organic Sulphates by Gluco-sulfatase. The solution of soluble organic sulphate just above mentioned was treated with barium chloride to remove inorganic sulphate and the filtrate was mixed with enzyme obtained from the liver of *Charonia* as follows:

	A	B
Extract of enzyme	10 c.c.	10 c.c.
Filtrate (substrate)	50 c.c.	—
Water+$BaCl_2$	—	50 c.c.
4/10 N-Na acetate	10 c.c.	10 c.c.
Chloroform	1 c.c.	1 c.c.

At the start there was 7.16 mg. S of organic sulphate in A. After standing for a week at 32–33°C., both were centrifuged and the precipitates of barium sulphate formed were separately collected, washed and ignited.

Found: 7.6 mg. $BaSO_4$ in A; 3.7 mg. $BaSO_4$ in B. Therefore 3.9 mg. $BaSO_4$ were produced from substrate by enzyme action.

The action of enzyme upon insoluble organic sulphate was examined as follows: 10 gr. of the insoluble part of *Charonia* (containing 19.1 mg. $S_{(org.)}$, none of inorganic sulphate), prepared in the course of foregoing analysis, were kneaded with 20 c.c. of enzyme solution (aqueous extract of the liver of another *Charonia*, containing none of organic sulphate and 18.8 mg. $S_{(inorg.)}$) and the suspension was kept at 35–36°C. for 2 weeks with some chloroform. At the end of the experiment, there was an increase of inorganic sulphate in liquid portion (11.1 mg. S), that is to say, 58.2% of the insoluble organic sulphate were hydrolysed by the enzyme. Thus, the soluble and insoluble organic sulphates occurring naturally in the shell-fish are attacked by the enzyme.

Thanks are due to Dr. C. Hattori who kindly supplied us the sample of enzyme.

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