GLUCOSULFATASE. V.

By Tokuro SODA and Fujio EGAMI.

Received April 18th, 1933. Published May 28th, 1933.

Glucosulfatase[1] is regarded as an enzyme which hydrolyses glucose-monosulphate.[2] Some of its properties, however, are still ambiguous. For instance, we do not know if: (1) it is simply a hydrolysing enzyme, (2) it can be obtained in pure state and (3) it can be obviously distin-

(1) T. Soda and C. Hattori, this Bulletin, **6** (1931), 258.
(2) T. Soda, *Biochem. Z.* **135** (1923), 623; this Bulletin, **8** (1933), 37.

guished from phenosulfatase. In the present paper we have chiefly dealt with these questions.

As experimental materials, we used at first the internal organs of *viviparus japonicus* (*tanishi*)[3] and afterwards those of *charonia lampas* (*boshu-bora*),[4] the liver of which is found to be the best material.[5]

Experiment A. I. The effect of the time of autolysis and the effect of $CaCO_3$ as a neutralising agent.

Enzyme solution. (a) Ten grams of the internal organs of *viviparus japonicus* dried with acetone were autolysed for 3 days in 60 c.c. of water at 35°C. in the presence of a little chloroform (we used it always as an antiseptic during the autolysis). The enzyme solution was then separated with a centrifuge. (b) Forty grams of the enzyme material were autolysed for 3 hours in 240 c.c. of water and the enzyme solution was obtained in the same way as in (a). Substrate solution. Solution of potassium glucose-monosulphate, 5 c.c. of which gave 0.0999 gr. $BaSO_4$.

The compositions of the reaction mixture and of the controls were as follows:

Reaction mixture:		Enzyme solution	50 c.c.
		Substrate solution	50
		Chloroform	2
Controls:	(1)	Enzyme solution	50 c.c.
		Chloroform	1
		With or without $CaCO_3$	
	(2)	Substrate solution	50 c.c.
		Chloroform	1
		With or without $CaCO_3$	

These solutions were kept at room temperature (ca. 20°C.) and the controls (1) and (2) were mixed just before it was put to analysis.

The method of estimation: 10 c.c. of the reaction mixture (or 5 c.c. of each control) were mixed with 10 c.c. of 20% trichloroacetic acid and the protein-precipitate was separated with a centrifuge. Fifteen c.c. of the clear solution were mixed then with 10 c.c. of 5% $BaCl_2$ solution and 15 c.c. of 5% KOH solution. It was immediately separated again from the precipitate in the same way as before. Now 30 c.c. of the clear solution were hydrolysed by HCl and thus the substrate, which had not been previously decomposed by the enzyme, was determined in the form of $BaSO_4$ by the ordinary gravimetric method. Result of experiments:

(3) T. Soda and C. Hattori, *J. Chem. Soc. Japan*, **54** (1933), 59.
(4) „ „ , this Bulletin, **8** (1933), 65.
(5) „ „ , *J. Chem. Soc. Japan*, **54** (1933), 377.

Table 1.

	Enzyme	$CaCO_3$	1 day	5 days	9 days	13 days
No. 1.	a	with	29.1%	44.7%	44.7%	46.0%
No. 2.	b	with	5.6	22.9	37.3	43.6
No. 3.	b	without	9.0	25.0	26.2	26.2
Control	b	with	—	—	—	0.4
,,	b	without	—	—	—	0.2

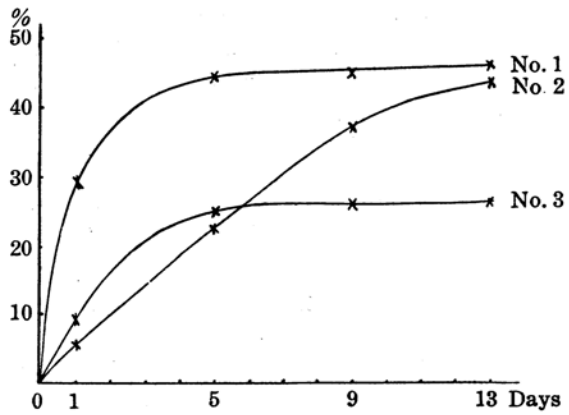

Fig. 1.

The percentage in this table expresses the rate of decomposition of the substrate. From these experiments we see that the enzyme solution (a) (autolysed for 3 days) was in the beginning much more active than that of (b) (autolysed for 3 hours). And we see from No. 2. and No. 3. that $CaCO_3$ is available for this kind of experiment as a neutralising agent.

II. The evidence of simply hydrolytic nature of the enzyme.

In order to ascertain that this enzyme actually hydrolyses glucose-monosulphate in the following way:

$$C_6H_{11}O_5 \cdot O \cdot SO_3K + H_2O = C_6H_{12}O_6 + KHSO_4$$

and has no other action than this, we tried to estimate the quantity of glucose produced by glucosulfatase. For this purpose the glucose thus produced was fermented by yeast and the volume of CO_2 obtained was approximately determined with the apparatus of Lunge and Rittener.[6]

(6) *Z. angew. Chem.*, **19** (1906), 1849.

Now, if we assume that the hydrolysis by the enzyme and the fermentation by yeast have been complete, 10 c.c. of the reaction mixture in the above experiment (I) would give at normal state

$$2\times 22412\times\frac{0.0999}{BaSO_4}=\frac{2\times 22412\times 0.0999}{233.5}=19.18\text{ c.c. of }CO_2$$

according to the equation of ordinary fermentation:

$$C_6H_{12}O_6 = 2C_2H_5OH + 2CO_2$$

As for the blank test, we have ascertained that the mixture of 5 gr. yeast, 5 c.c. enzyme solution and 45 c.c. of water, on standing, evolved no CO_2. From the mixture of 5 gr. yeast, 10 c.c. of sample No. 2. in the forgoing article (hydrolysed for 9 days) and 40 c.c. water evolved 7.83 c.c. CO_2, at the atm. pressure (P) = 757.8 mm., the temperature (t) = 22.3°C. and vapour tension of water (p) = 20.3 mm. The volume of CO_2 at N.S. (V) has been calculated from the observed volume (v) by the following formula:

$$V = v\frac{273.1}{273.1+t}\cdot\frac{760}{P-0.8p}\ ^{(7)}$$

From the above data we have V = 7.06 c.c. Therefore the percentage of glucose hydrolysed by the enzyme can be calculated as follows:

$$7.06/19.18\times 100 = 36.8\%\ .$$

The percentage obtained from the determination of SO_4 = 37.3%, as is shown in Table 1. This coincidence of both percentages could be regarded as the proof that glucosulfatase is simply a hydrolysing enzyme. After 20 days the same experiment as above was repeated. The blank test, however, gave this time 2.29 c.c. of CO_2 therefore this amount was subtracted from the volume 16.65 c.c. of CO_2 evolved by fermentation, and we obtain v = 14.36 c.c. at t = 25.5°, P = 756.2 mm., p = 24.5 mm. Consequently V = 12.73 c.c., and the enzymic hydrolysis expressed in percentage is $12.73/19.18\times 100 = 66.4\%$. The corresponding percentage calculated from the determination of SO_4 is 64.2%.

Experiment B. Adsorption and purification of the enzyme.

The dry enzyme preparations were made from *charonia lampas* in the same way as in Experiment A. The substrate solution was either

(7) The factor "0.8" in this formula is to reduce the vapour pressure of pure water to that of saturated NaCl-solution or of KOH-solution (1: 2).

sodium or barium salt of glucose-monosulphate of 0.0709 N.[8] The experiments were always carried out at 30.0°C. in a thermostat.

I. Adsorption by kaolin.

Enzyme solution: No. 1. 20 Gr. of the dry enzyme preparation were autolysed for a day in 100 c.c. of water at 32°C. and the clear enzyme solution was separated with a centrifuge. No. 2. 8 Gr. of kaolin were added to 25 c.c. of the above enzyme solution (No. 1), and it was clarified again as above and made up to 25 c.c. by adding water to it (kaolin here used was that of Merck heated for about 2 hours at 200°C.).

Substrate solution:		Solution of sodium glucose-monosulphate.	
Reaction mixture:		Substrate solution	30 c.c.
		Enzyme solution	20
		Water	10
		Chloroform and $CaCO_3$	
Controls:	(1)	Substrate solution	30 c.c.
		Chloroform and $CaCO_3$	
	(2)	Enzyme solution	20 c.c.
		Water	10
		Chloroform and $CaCO_3$	

The experiment procedure: same as is described in Experiment A. Result of experiments:

Enzyme	1 day	2 days
No. 1.	13.4%	20.1%
No. 2.	1.3	1.5
Control (No. 1.)	—	3.6

We see that the enzyme was almost perfectly adsorbed by kaolin. The 3.6% hydrolysis observed in the control is probably due to the presence of some unknown ethereal sulphate in the enzyme preparation.

Two c.c. of both enzyme solutions were dried separately in vacuum over $CaCl_2$. The weight of No. 1. and No. 2. after being dried were 0.0918 gr. and 0.0324 gr. respectively. From these data we have attempted to calculate the concentration of glucosulfatase. Assuming that the reaction proceeds as monomolecular (on this point we shall deal later on) the velocity constant (k) was calculated as follows:

$$k = \frac{1}{t}\log\frac{a}{a-x} = \log\frac{100}{100-13.4} = 0.0625 \quad \text{(Enzyme No. 1.)}$$

(8) Concentration of substate solutions is always of the same normality if not otherwise stated.

As 20 c.c. of enzyme solution were diluted with 10 c.c. of water, the actual dried weight of 2 c.c. of the enzyme solution should be

$$m = 0.0918 \times 2/3 = 0.0612 \text{ gr.}$$

If we express the concentration of our glucosulfatase as "G-Sulf." (Glucosulfatase-fähigkeit), so the concentration of Enzyme No. 1. is: G-Sulf $= k/m = 1.0$; Enzyme No. 2.: G-Sulf $= 0.3$.

II. Adsorption experiments with $BaSO_4$ and aluminium hydroxide gel.[9]

Enzyme solution: No. 1. 15 Gr. of the dry enzyme preparation were autolysed for 2 days in 80 c.c. of water at 32°C. and the enzyme solution was separated with a centrifuge.

No. 2. Ten c.c. of aluminium hydroxide gel were added to 25 c.c. of the enzyme solution No. 1, and the clear enzyme solution No. 2 was separated by means of a centrifuge.

No. 3. Five c.c. of 10% $BaCl_2$ solution were added to 25 c.c. of the enzyme solution No. 1 which contained some inorganic sulphate. Then the enzyme solution No. 3 was separated from the precipitate (which contained proteins and $BaSO_4$) with a centrifuge.

Substrate solution:	Solution of sodium glucose-monosulphate.	
Reaction mixture:	Substrate solution	20 c.c.
	Enzyme solution	20
	Chloroform and $CaCO_3$	

Result of experiments:

Enzyme	Rate of hydrolysis in 2 days	Weight of 2 c.c. enzyme solution dried	Concentration of enzyme (G-Sulf)
No. 1.	24.4%	0.0595 gr.	1.0
No. 2.	22.7	0.0398	1.4
No. 3.	75.7	—	—

From the above result, it is obvious that aluminium hydroxide gel adsorbs scarcely the enzyme, but it removes the impurities fairly well.

(9) Preparation of aluminium hydroxide gel: saturated solution of $AlCl_3.6H_2O$ (20 gr.) was added to 500 c.c. of warm ammonium hydroxide solution (5%). Then the solution was boiled for about 7 hours keeping its volume as constant as possible. It was then dialysed for several weeks. Two c.c. of the gel thus obtained, after being dried in vacuum over $CaCl_2$, weighed 0.0138 gr., and after ignition it weighed 0.0091 gr. as Al_2O_3.

The most remarkable fact is the increase of activity by the treatment with $BaCl_2$ (Enzyme No. 3). We have, therefore, studied the purification of the enzyme by applying these two methods and found very effective as is described in the next article.

III. Purification of the enzyme with $BaCl_2$ and $Al(OH)_3$ gel.

Enzyme solution: 10 Gr. of the dry enzyme preparation were autolysed for 2 days in 50 c.c. of water at 32°C. Then it was separated with a centrifuge. Ten c.c. of 10% $BaCl_2$ solution were added to it and again separated as before. The solution was then treated with 5 c.c. of $Al(OH)_3$ gel and subsequently centrifuged. A quite clear enzyme solution was thus obtained.

Substrate solution:	Solution of barium glucose-monosulphate with an excess of $BaCl_2$.	
Reaction mixture:	Substrate solution	20 c.c.
	Enzyme solution	20
	Chloroform and $CaCO_3$	

The method of estimation: As the enzyme solution thus obtained was almost free from proteins, the treatment with trichloroacetic acid was omitted and the procedure was simplified as follows:

Ten c.c. of the filtered reaction mixture were decomposed by HCl and the amount of substrate was determined as usual in the form of $BaSO_4$. Result of the experiment: The amount of hydrolysis in a day $= 82.0\%$, $k = 0.745$; the weight of 2 c.c. enzyme solution dried = ca. 0.11 gr. (in which some excess of $BaCl_2$ is contained; therefore the original weight must be less than this). From these data we have: G-Sulf. = 7.

IV. Adsorption experiment with charcoal.

Enzyme solution: 30 Gr. of the dried liver were autolysed for 2 days in 120 c.c. water at 32°C. It was separated with a centrifuge and 4 c.c. of Na_2SO_4 solution (saturated at 15°C.) and 12 c.c. of 10% solution of $BaCl_2$ were successively added to it. Then it was centrifuged again. Ten c.c. of $Al(OH)_3$ gel were added to it. After centrifuging off the gel, the solution was treated with 5 gr. of charcoal and subsequently filtered. This enzyme solution was perfectly clear.

Substrate solution:	Solution of barium glucose-monosulphate with an excess of $BaCl_2$.	
Reaction mixture:	Substrate solution	30 c.c.
	Enzyme solution	30
	Chloroform and $CaCO_3$	

Result of the experiment:

The rate of hydrolysis in a day = 50.1 %
The velocity constant k = 0.302
The weight of 2 c.c. enzyme solution dried = 0.0378 gr.
,, ,, ,, ash ($BaCl_2$, NaCl etc.) = 0.0051
,, ,, ,, enzyme, proteins and unknown org. substances = 0.0327

Hence G-Sulf = 0.302 / 0.0327 = 9.5

Fifty c.c. of this enzyme solution were dialysed for 2 days through a collodion membrane and was precipitated in acetone and dried over $CaCl_2$ in vacuum. The yield was ca. 0.2 gr. It was readily and perfectly soluble in water. Fifteen c.c. of the substrate solution was hydrolysed by 0.0644 gr. of this preparation dissolved in 15 c.c. of water. The hydrolysis proceeded 25% in a day. Hence

$$\text{G-Sulf} = \frac{1}{0.0644 \times 2/15} \log \frac{100}{100-25.0} = 11.6$$

We have thus obtained a solid preparation of glucosulfatase which is 11.6 times more concentrated than the original enzyme.

Experiment C. I. Effects of glucose and galactose upon enzyme action.

Enzyme solution: 20 Gr. of internal organs of *viviparus japonicus* dried with acetone were autolysed for a week in 100 c.c. of water at room temperature (20–28°C.) and centrifuged.

Substrate solution: Solution of potassium glucose-monosulphate, 5 c.c. of which gave 0.0999 gr. $BaSO_4$.

Reaction mixtures: No. 1. Enzyme solution 20 c.c.
Water 10
Substrate solution 30
Chloroform and $CaCO_3$

No. 2. 10 c.c. of glucose solution (2.446 gr. glucose in 100 c.c.) instead of water in No. 1.

No. 3. 10 c.c. of galactose solution (2.450 gr. in 100 c.c.) instead of water in No. 1.

Results of experiment:

	1 day	2 days	5 days	10 days
No. 1.	3.0%	7.3%	16.3%	23.1%
No. 2.	1.0	2.7	10.7	13.6
No. 3.	1.0	2.8	10.8	14.5

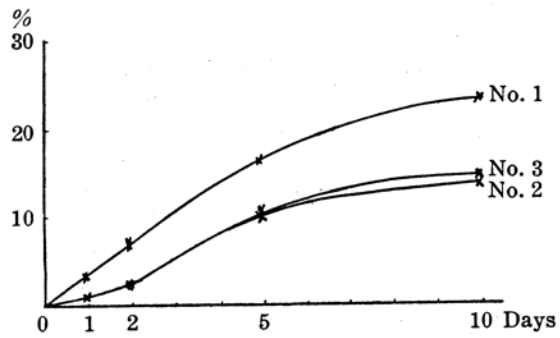

Fig. 2.

According to these results, glucose and galactose inhibit the action of glucosulfatase and indeed in about same degree.

II. Effects of inorganic sulphate and phosphate and magnesium carbonate.

The inhibiting action of phosphate upon phosphatase has been studied by many biochemists. Recently E. Jacobsen[10] studied also this problem and he explained the mechanism of this inhibition by the affinity between the phosphate and the enzyme. Therefore it would be interesting to study the action of sulphate ion upon glucosulfatase in the analogous way.

According to C. Hommerberg,[11] phosphate has also inhibiting action upon phenosulfatase and therefore we must remove phosphate ion, if we want to obtain very active phenosulfatase.

The effect of magnesium salt, an activator of phosphatase,[12] upon phenosulfatase has also been studied. Neuberg and Linhardt[13] tried to use $MgCO_3$ instead of $CaCO_3$ as a neutralising agent, whereas C. Hommerberg[14] used $MgCl_2$ in order to examine the effect of Mg-ion. According to them, Mg-ion has always a remarkable inhibiting action upon phenosulfatase. It would be, therefore, also significant to examine the actions of phosphate and $MgCO_3$ upon glucosulfatase.

Enzyme solution: 20 Gr. of the dry liver of *charonia lampas* were autolysed for 2 days in 80 c.c. of water at 32°C. Crude enzyme solution was separated with a centrifuge and 4 c.c. of Na_2SO_4 solution and 12 c.c.

(10) *Biochem. Z.*, **249** (1932), 21.
(11) *Z. physiol. Chem.*, **200** (1931), 79.
(12) Erdtman, *Z. physiol. Chem.*, **177** (1928), 211.
(13) *Biochem. Z.*, **142** (1923), 192.
(14) *Z. physiol. Chem.*, **200** (1931), 80.

of 10% $BaCl_2$ solution were successively added. The precipitate was centrifuged off and the solution was shaken with 2 gr. of charcoal, thereafter it was filtered and dialysed.

Substrate solution:	Solution of sodium glucose-monosulphate.	
Reaction mixtures:	Substrate solution	30 c.c.
	Enzyme solution	10
	Buffer solution (acetate buffer, pH: 7)	10
	Water	5
	1. Water	5
	or (a) Sulphate solution: 12% Na_2SO_4	
	or (b) Phosphate solution: $NaH_2PO_4+Na_2HPO_4$, pH = 7; 6% as P_2O_5	
	2. Water and $CaCO_3$ or Water and $MgCO_3$	5

Method of estimation: Ten c.c. of the reaction mixture were treated with 6 c.c. of 10% $BaCl_2$ solution and 4 c.c. of 1% KOH solution and it was then filtered. Fifteen c.c. of the filtrate were put to analysis as already described (Experiment B, III).

Results of experiments:

		The rate of hydrolysis in 2 days
1.	Water	27.6%
	Sulphate (a)	13.6
	Phosphate (b)	0.0
2.	$CaCO_3$	16.6
	$MgCO_3$	9.2

The inhibiting action of phosphate is emormously strong; that of sulphate and $MgCO_3$ is also evident.

Experiment D. On glucosulfatase and phenosulfatase in the liver of *charonia lampas*.

I. Comparison of the activities of glucosulfatase and phenosulfatase.

Enzyme solution: 10 Gr. of the liver dried with acetone were autolysed for 2 days in 50 c.c. of water at 32°C., and from this the enzyme solution was prepared after the procedure described in Experiment B, III.

Substrate solutions: No. 1. solution of barium glucose-monosulphate with an excess of $BaCl_2$. No. 2. solution of sodium phenolsulphate with an excess of $BaCl_2$. Both solutions had the same normality. (0.0709 N).

Reaction mixture:	Enzyme solution	20 c.c.
	Substrate solution	20 c.c.
	Chloroform and $CaCO_3$	

Result of experiment:

Substrate	The rate of hydrolysis in a day
No. 1. (Gl)	78.8%
No. 2. (Ph)	75.6

Neuberg and Simon[15] as well as Hommerberg[16] tried to prepare phenosulfatase from some mammalian organs. The former could extract the enzyme but with only weak activity (8% hydrolysis in several days), while the latter did not succeed to prepare it. Phenosulfatase obtained from the liver of *charonia lampas* has probably the strongest activity ever extracted from animal organs (almost comparable to that of Taka-diastase).

II. Adsorption of glucosulfatase and phenosulfatase by kaolin.

Enzyme solution: No. 1. 30 Gr. of the dried liver were autolysed for 2 days in 120 c.c. of water 32°C. and further treated as in Experiment B, III (10% $BaCl_2$: 20 c.c., $Al(OH)_3$: 20 c.c.).

No. 2. 15 Gr. of kaolin were added to 60 c.c. of the enzyme solution (No. 1.) and it was separated with a centrifuge. The clear solution was diluted with water to 60 c.c.

No. 3. The kaolin which adsorbed the enzyme was washed with water.

Substrate solutions: No. 1. solution of barium glucose-monosulphate with an excess of $BaCl_2$. No. 2. solution of sodium phenolsulphate with an excess of $BaCl_2$.

Reaction mixtures:

No. of enzyme	No. 1.	No. 2.	No. 3.
Substrate solution	70 c.c.	20 c.c.	20 c.c.
Enzyme solution	70	20	20 c.c. water+6 gr. kaolin with enzyme

Chloroform and $CaCO_3$ were added.

Result of experiments:

(15) *Biochem. Z.*, **156**(1925), 372.
(16) *Z. physiol. Chem.*, **200**(1931), 69.

Subst.	Enzyme	3 hours	6 hours	21 hours	58 hours
(a) No. 1.	No. 1.	17.6%	33.1%	73.9%	75.1%
(b) No. 1.	No. 2.	—	—	—	1.6
(b) No. 2.	,,	—	—	—	2.5
(c) No. 1.	No. 3.	—	—	—	29.5
(c) No. 2.	,,	—	—	—	49.3

The reaction velocity constants calculated as monomolecular reaction from data of experiment (a) are:

Hours	3	6	21	58
k	0.028	0.029	0.028	0.012

Therefore the reaction is monomolecular at least for first 20 hours. Both enzymes are adsorbed well by kaolin with about the same ratio as in the solution. It seems hopeless to separate these two enzymes by the adsorption method such as described above.

III. Evidence that the phenosulfatase and the glucosulfatase are not one and the same enzyme.

We have seen that the glucosulfatase always hydrolyses also phenolsulphate. We do not know, however, whether the glucosulfatase can by itself hydrolyse phenolsulphate or it is always mixed with the phenosulfatase which had been studied by Neuberg and his co-workers. In this experiment, we attempted to distinguish these two enzymes by their different stability against weak alkali and also by the inhibiting action of glucose upon both enzymes.

Enzyme solution: The enzyme solution (A) was prepared in the same way as described in Experiment C, II, but without dialysis.

No. 1. Forty c.c. of the enzyme solution (A) were diluted with 4 c.c. of water. No. 2. To 20 c.c. of the enzyme solution (A) 1 c.c. of 2 N. ammoniumhydroxide solution was added and kept for 16 hours. Then it was neutralised by 2 N. acetic acid.

Substrate solution: solutions of barium glucose-monosulphate and sodium phenolsulphate both with an excess of $BaCl_2$.

Reaction mixture:		
	Substrate solution	15 c.c.
	Enzyme solution	10 c.c.
	Water or glucose solution (10%)	5 c.c.
	Chloroform and $CaCO_3$	

Results of experiment:

Substrate	Enzyme	Water or glucose soln.	The rate of hydrolysis in a day
Glucose-sulphate	No. 1.	water	70.1%
,,	No. 2.	,,	13.6
,,	No. 1.	glucose	61.1
Phenol-sulphate	No. 1.	water	29.0%
,,	No. 2.	,,	36.6
,,	No. 1.	glucose	27.8

The above data show that the action of glucosulfatase is strongly inactivated by weak alkali while that of phenosulfatase is rather activated by it. Moreover, glucose which has remarkable inhibiting action upon glucosulfatase scarcely inhibits the action of phenosulfatase. These facts may serve for the evidence that glucosulfatase and phenosulfatase are not one and the same enzyme.

The difference of activities of glucosulfatase and phenosulfatase in this experiment is very remarkable compared with that observed in Experiment D, I. Charcoal seems to adsorb phenosulfatase.

Summary.

1. Glucosulfatase is a simply hydrolysing enzyme.
2. It is almost perfectly adsorbed by kaolin.
3. It can not be adsorbed by $BaSO_4$, aluminium hydroxide gel and charcoal. These adsorbents can be used for the purification of the enzyme. Thus a perfectly clear enzyme solution is obtainable. A solid preparation of very active glucosulfatase was obtained. Its purity (G-Sulf) is 11.6 while the original value was 1.0.
4. The enzyme action is inhibited by: glucose, galactose, $MgCO_3$, inorganic sulphate and phosphate.
5. Glucosulfatase always contains phenosulfatase but they are not one and the same enzyme.

Chemical Laboratory, Faculty of Science,
Tokyo Imperial University.