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Effects of some Heavy-Metal Ions on Purified Mammalian β-Glucuronidase

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Heavy-metal ions and organic mercurials are known to have a pronounced inhibitory action on many enzymes (see Dixon & Webb, 1958). It is generally accepted that these reagents are reactive towards sulphydryl groups (see Gurd & Wilcox, 1956; Cecil & McPhee, 1959; Boyer, 1959) and also towards other groups (Sohler, Seibert, Kreke & Cook, 1952; Myrbäck, 1957). A detailed investigation of the effects of heavy-metal ions on a well characterized purified enzyme could lead to a better understanding of both their mode of action and the nature of the active centres in the enzyme.

β-Glucuronidase from various sources is inhibited by fairly low concentrations (less than mM) of mercuric, silver and cupric ions (Sarkar & Sumner, 1950; Jarrige & Henry, 1952; Doyle, Katzman & Doisy, 1955; Levvy, Hay & Marsh, 1957), whilst being unaffected by anionic enzyme poisons such as fluoride, cyanide or iodoacetate (Sarkar & Sumner, 1950; Wong & Rossiter, 1951). The general properties of the enzyme have recently been reviewed by Levvy & Marsh (1959, 1960), who have also studied the effects of heavy-metal ions on rat- and mouse-liver β-glucuronidase (Levvy & Marsh, 1957*a*, *b*). The present paper is based on similar experiments with a highly purified mammalian β-glucuronidase.

MATERIALS AND METHODS

Enzyme. β-Glucuronidase was extracted from female-rat preputial gland and purified according to the procedure of Levvy, McAllan & Marsh (1958). Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystallized bovine plasma albumin (L. Light and Co. Ltd.) as standard. The results were largely obtained from a single preparation whose specific activity was initially 500 000 units/mg. of protein. One glucuronidase unit liberates 1 μg. of phenolphthalein in 1 hr. from 0·63 mM-phenolphthalein β-glucuronide in 0·05 M-acetic acid–NaOH buffer, pH 4·5, at 38° in the presence of 0·01% of albumin. A solution of the enzyme (1 mg. of protein/ml.) in 0·1 M-acetic acid–NaOH buffer, pH 5·2, was stable for at least 18 months at 0°, during which time the specific activity rose slightly in association with the separation of inert material. This enzyme has not so far been crystallized, though its activity is at least four times that of a crystalline specimen of *Helix pomatia* β-glucuronidase prepared by Alfsen & Jayle (1958).

Chemicals. Phenolphthalein β-glucuronide was obtained biosynthetically as described by Levvy & Marsh (1959). Phenyl β-glucuronide dihydrate, m.p. 160°, was prepared by catalytic oxidation of phenyl β-glucoside (Marsh & Levvy, 1958). Aryl glycosides were kindly provided by Dr J. Conchie, Rowett Research Institute. Phenylmercuric hydroxide, m.p. 226°, was prepared from the nitrate (Schramm, 1947). All others were commercial preparations.

General assay procedure. Incubations with phenolphthalein β-glucuronide were performed at 38° in duplicate 15 ml. Pyrex conical centrifuge tubes, each containing 4·0 ml. of liquid. Stock enzyme solution was diluted before use with an albumin solution: this protected and activated the otherwise labile enzyme in dilute solution. The use of Triton X-100 as a substitute for albumin has been suggested by Woollen, Heyworth & Walker (1961), but even 0·1% concn. was not as effective as 0·01% of albumin. Concentrations of reagents are final in the assay mixture (4·0 ml.) unless otherwise indicated. Enzymic hydrolysis was arrested by addition of 4·0 ml. of 0·4 M-glycine–NaOH buffer, pH 10·7, and the quantity of phenolphthalein released (up to 80 μg.; Levvy & Marsh, 1959) was measured colorimetrically with a Hilger Spekker absorptiometer (Ilford no. 605 filters, peak transmission 550 mμ).

With phenyl β-glucuronide as substrate, a somewhat different procedure was adopted (Levvy & Marsh, 1959). The final assay volume was 1·0 ml. and the reaction was terminated by addition of 2·5 ml. of freshly diluted (1:5) Folin–Ciocalteu reagent. After centrifuging, a sample (2·5 ml.) was mixed with 5·0 ml. of N-Na_2CO_3 and the colour was developed at 38° for 20 min. The quantity of phenol released (up to 100 μg.; Levvy & Marsh, 1959) was similarly measured colorimetrically (Ilford no. 608 filters, peak transmission 680 mμ). The results are expressed as final concentrations (μM) of phenolphthalein or phenol present in the reaction mixture. Details of the various procedures are as follows.

(*a*) Velocity curves. To the enzyme, in 0·01% of albumin and 0·05 M-acetic acid–NaOH buffer at the pH of assay, inhibitor was added. This mixture was preincubated for 10 min. at 38° before addition of substrate and subsequent incubation times varied from 15 to 120 min. In reversal experiments, reversing agents were added after incubation of enzyme, inhibitor and substrate for 30 min.

(*b*) Effect of preincubation. Buffer, enzyme–albumin and inhibitor were preincubated at 38° for varying lengths of time (1–120 min.) before addition of substrate and subsequently for 60 min. In suppression experiments, the suppressing agents and substrate were added 10 min. and 20 min. respectively after addition of inhibitor.

(*c*) Effect of inhibitor concentration. Buffer, enzyme–albumin and inhibitor were preincubated for 10 min. before addition of substrate and subsequently for 60 min.

(*d*) Effect of substrate concentration. Procedure was as in (*c*). Phenolphthalein β-glucuronide concentrations ranged from 10 μM to 2·5 mM; phenyl β-glucuronide concentrations ranged from 100 μM to 10 mM.

(*e*) Effect of pH. Procedure was as in (*c*). With phenolphthalein β-glucuronide as substrate, final pH values, after addition of glycine–NaOH buffer, were adjusted to 10·7 with predetermined amounts of 0·5N-NaOH and volumes were made up to 10·0 ml. with water. Alternatively, with multiple measurements at each pH, a series of calibration curves was drawn up, so obviating any pH adjustments. Both these procedures were unnecessary with phenyl β-glucuronide as the estimation of phenol was not pH-dependent.

Cuprous ion determinations (*Frieden*, 1958). The yellow colour developed with 0·5 mM-2,9-dimethyl-1,10-phenanthroline in 0·05M-acetic acid–NaOH buffer, pH 4·5, was proportional to the concentration of cuprous copper (1–100 μM; Spekker with Ilford no. 601 filters, peak transmission 430 mμ).

Experiments with cuprous chloride. A solution of 0·2M-CuCl in 2N-HCl, stored under N_2, was used in conjunction with an all-glass Agla micrometer syringe. Purification of reagents to decrease the concentration of endogenous copper below 0·1 μM involved the extraction of buffer solutions with dithizone (0·01% in $CHCl_3$) and storage in polythene containers. All solvents were redistilled and glassware was subjected to a rigorous washing procedure, HNO_3–H_2SO_4 (1:1, Thiers, 1957) being used for the initial cleaning.

Hydrolysis of aryl glycosides by cuprous copper. In the course of this work it was observed that ascorbate (or dihydroxymaleate) in the presence of Cu^{2+} ions seriously interfered with the actual estimation of β-glucuronidase activity, in that phenolphthalein was released from the glucuronide even in the absence of enzyme. This effect in part opposed the inhibitory action of copper and ascorbate on the enzyme in the net result of the enzyme assay.

Phenolphthalein was identified by paper chromatography (butan-1-ol satd. with aq. 5N-NH_3 soln.) of the ether-soluble material present after the following reaction, which also produced brownish pigments (after colour development at pH 10·7) not extractable into ether at pH 5·2. A 1·5% hydrolysis was achieved by addition of mM-ascorbate to mM-phenolphthalein β-glucuronide and 0·1 mM-Cu^{2+} ions in 0·05M-acetic acid–NaOH buffer, pH 5·2, with incubation of the mixture at 38° for 60 min. Copper was essential, but only traces (μM) were required, and in many cases there was sufficient endogenous copper in the system to cause appreciable colour development. In the absence of oxygen, the yield of all pigments was considerably diminished.

The effect of Cu^{2+} ions and ascorbate on several aryl glycosides, namely *p*-nitrophenyl α-mannoside, *o*-nitrophenyl β-galactoside, phenyl α-*N*-acetylglucosaminide and *p*-nitrophenyl β-*N*-acetylglucosaminide, was examined and in each case significant hydrolysis occurred under conditions employed for glycosidase studies (Conchie, Findlay & Levvy, 1959), although no enzyme was present. With the last of these compounds it was possible to measure both the *p*-nitrophenol and *N*-acetylglucosamine (Levvy & McAllan, 1959) produced in the reaction and these were found to be equimolar. In view of these findings, it is important that suitable controls should be included when using reagents such as ascorbate.

In the above-mentioned examples copper was probably acting catalytically, but with phenolphthalein β-glucuronide at least a similar effect (without production of brown pigments) was achieved by adding a solution of CuCl (5 mM) or by shaking with solid (10 mM). In the latter case, with 0·5 mM-substrate, 6% was converted into phenolphthalein in the presence of air and only 2% under an atmosphere of N_2. It is thought that Cu^+ ions are involved in this hydrolytic fission and that O_2 is an enhancing factor.

RESULTS

The effects of Cu^{2+}, Ag^+ and Hg^{2+} ions, *p*-chloromercuribenzoate and phenylmercuric hydroxide on purified β-glucuronidase have been investigated regularly by introducing a series of variables. Those under consideration were time of contact between enzyme and inhibitor both in the presence and absence of substrate, inhibitor concentration, substrate and substrate concentration and pH of incubation. The general procedure adopted was, as far as possible, to study only one variable at a time. Enzyme concentration was not regarded as being a contributory factor because the inhibitor concentrations concerned were not of the same order as the enzyme concentration (about 0·03 μg. of protein/ml.) and because assays were normally performed in the presence of 0·01% of albumin (100 μg./ml.), making the enzyme a negligible fraction of the total protein.

Time-course of reaction. Velocity curves for Hg^{2+} and Ag^+ ions are illustrated in Fig. 1. It is apparent that the curves are non-linear (due allowance being made for the two non-linear controls), the inhibitions increasing with increasing incubation time. Furthermore the non-linearity is more pronounced at high substrate concentration both with phenolphthalein β-glucuronide and phenyl β-glucuronide. Curves in the presence of 0·04 mM-*p*-chloromercuribenzoate and 0·25 mM-phenylmercuric hydroxide were linear (0·4 mM-phenolphthalein β-glucuronide and 0·05M-acetic acid–NaOH buffer, pH 4·5) and with 0·63 mM-Cu^{2+} ions a slight progressive inactivation modified the otherwise linear curve (0·63 mM-phenolphthalein β-glucuronide and 0·05M-acetic acid–NaOH buffer, pH 5·9).

Preincubation effects are shown in Table 1. At the concentrations normally employed in inhibition experiments, inactivation by heavy-metal ions is not generally more than 10–15%/hr. of preincubation. At higher concentrations, inactivation may be a much more important factor; thus 0·2 mM-Hg^{2+} ions produced 50% inactivation in 10 min. at 38°. Phenylmercuric hydroxide and *p*-chloromercuribenzoate appeared to have no inactivating action in this type of experiment.

Effect of inhibitor concentration. The various inhibition curves are shown in Fig. 2. Assay condi-

tions for Hg^{2+} and Ag^{+} ions (Fig. 2*a*) are optimum for the uninhibited enzymic hydrolysis of phenolphthalein β-glucuronide. Cupric ion is a very weak inhibitor at pH 4·5 (Fig. 5) and is more potent at pH 5·9, the upper limit for an acetic acid–NaOH buffer. In Fig. 2*b*, showing the effect of *p*-chloromercuribenzoate and phenylmercuric hydroxide, a substrate concentration equal to K_m was chosen because the effects of each compound can be interpreted on the basis of a single action and constants can be more readily derived with this substrate concentration (see below).

Effect of phenolphthalein β-glucuronide concentration. At pH 4·5, β-glucuronidase is normally inhibited by high concentrations of substrate (Fig. 3*a*), the behaviour being consistent with the formation of an inactive ES_2 complex, whose dissociation constant, K_{s_2}, is about 2 mM com-

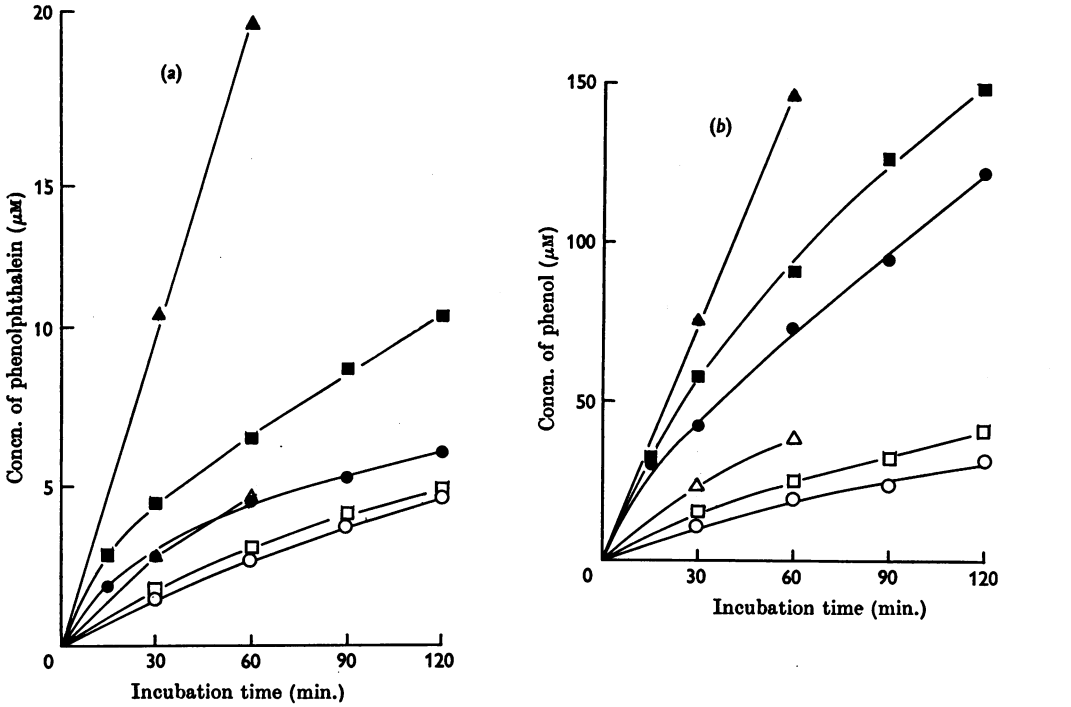

Fig. 1. (*a*) Velocity curves for the enzymic hydrolysis of 0·025 and 1·0 mM-phenolphthalein β-glucuronide, alone (△, ▲) and in the presence of 0·05 mM-$HgCl_2$ (○, ●) and 0·15 mM-$AgNO_3$ (□, ■). Buffer system was 0·05 M-acetic acid–NaOH, pH 4·5. (*b*) Velocity curves for the enzymic hydrolysis of 0·4 and 2·5 mM-phenyl β-glucuronide, alone (△, ▲) and in the presence of 0·05 mM-$HgCl_2$ (○, ●) and 0·15 mM-$AgNO_3$ (□, ■). Buffer system was 0·05 M-acetic acid–NaOH, pH 4·5. For other details see the Materials and Methods section.

Table 1. *Preincubation of β-glucuronidase with heavy-metal ions at 38° before assay with 0·63 mM-phenolphthalein β-glucuronide*

Preincubations and assays were performed at the same pH in 0·05 M-acetic acid–NaOH buffer. Values are recorded as percentages of the initial activities (100% representing about 20 μM-phenolphthalein). Other details are given in the Materials and Methods section. CMB, *p*-chloromercuribenzoate; PMH, phenylmercuric hydroxide.

Preincubation time (min.)	Relative activities				
	0·04 mM-$HgCl_2$, pH 4·5	0·15 mM-$AgNO_3$, pH 4·5	0·63 mM-$CuSO_4$, pH 5·9	0·04 mM-CMB, pH 4·5	0·25 mM-PMH, pH 4·5
1	100	100	100	100	100
15	98	95	90	103	104
30	96	90	87	103	102
60	92	82	85	100	98
120	93	72	81	100	96

pared with K_m, about 80 μM. Values for K_m in acetate buffer at this pH (and lower) derived from reciprocal plots (Lineweaver & Burk, 1934) tended to give erroneous values due to high percentage hydrolysis at one extreme and substrate inhibition at the other, and use of the integral Michaelis equation (Dixon & Webb, 1958) was found to be more satisfactory.

Mercuric and Ag^+ ions produce a simulated non-competitive inhibition at low substrate concentration with increasing inhibition at higher substrate

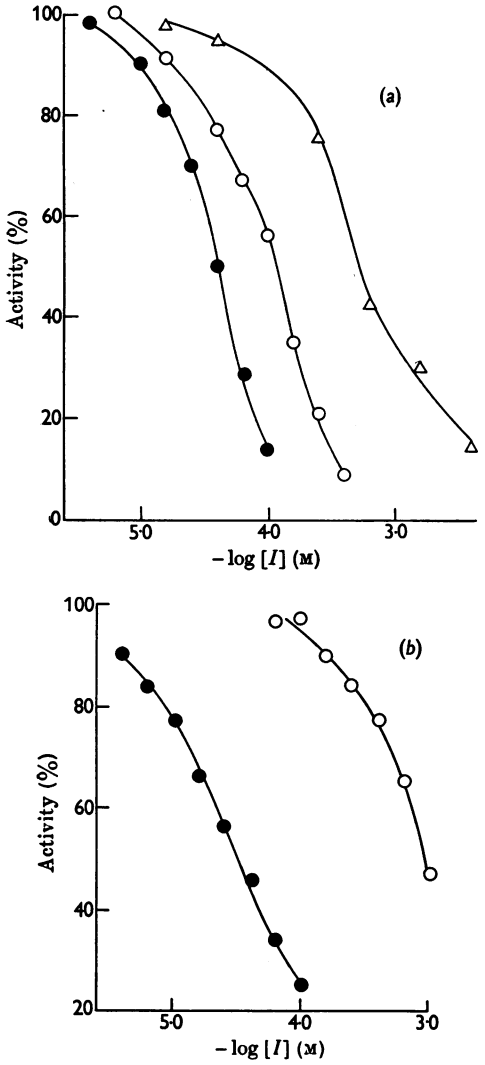

Fig. 2. (*a*) Inhibition of β-glucuronidase by $HgCl_2$ (●) and $AgNO_3$ (○) at pH 4·5, and by $CuSO_4$ (△) at pH 5·9, as a function of inhibitor concentration. Assays were performed in 0·05 M-acetic acid–NaOH buffer with 0·63 mM-phenolphthalein β-glucuronide (100% activity represents about 36 μM-phenolphthalein). (*b*) Inhibition of β-glucuronidase by *p*-chloromercuribenzoate (●) and phenylmercuric hydroxide (○) in the presence of 0·08 mM-phenolphthalein β-glucuronide and 0·05 M-acetic acid–NaOH buffer, pH 4·5 (100% activity represents 12 μM-phenolphthalein). For other details see the Materials and Methods section.

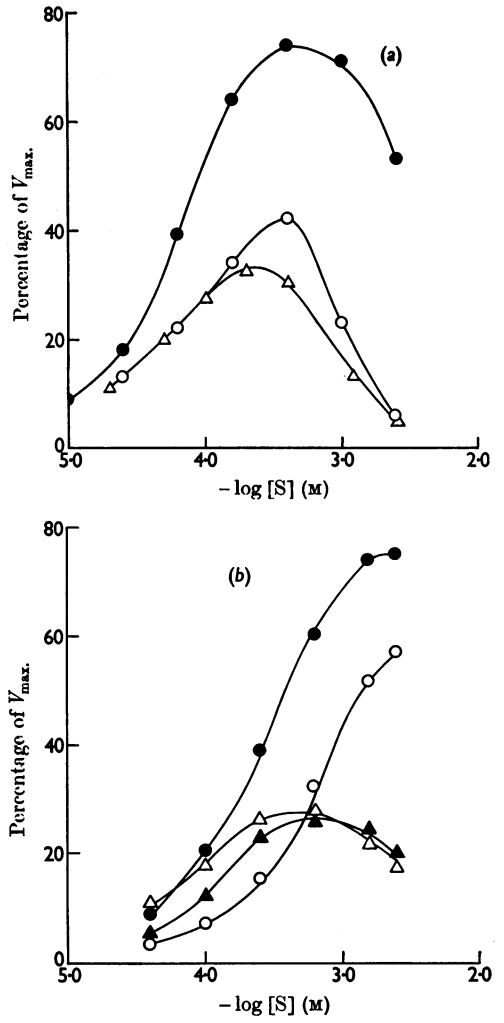

Fig. 3. (*a*) Inhibition of β-glucuronidase by 0·04 mM-$HgCl_2$ (○) and 0·15 mM-$AgNO_3$ (△) in 0·05 M-acetic acid–NaOH buffer, pH 4·5, as a function of phenolphthalein β-glucuronide concentration. The control series (●) is with substrate alone. $V_{max.}$ (48 μM-phenolphthalein) is the calculated maximum velocity for the control. (*b*) Inhibition of β-glucuronidase by 0·63 mM-$CuSO_4$ (▲), 0·04 mM-*p*-chloromercuribenzoate (○) and 0·25 mM-phenylmercuric hydroxide (△) in 0·05 M-acetic acid–NaOH buffer, pH 5·9, as a function of phenolphthalein β-glucuronide concentration. The control series (●) is with substrate alone ($V_{max.}$ 45 μM-phenolphthalein). For other details see the Materials and Methods section.

concentration (Fig. 3*a*). Cupric ion at pH 5·9, where normally K_m is about 400 μM and K_{S_2} 14 mM, increases inhibition by excess of substrate, i.e. causes a decrease in K_{S_2} (Fig. 3*b*). At low substrate concentration it is difficult to distinguish between competitive and non-competitive inhibitions, and constants for either case are compatible with the experimental results. Inhibition by *p*-chloromercuribenzoate is entirely competitive and the dissociation constant (K_i) is about 16 μM at pH 5·9. Phenylmercuric hydroxide inhibition is different in character and is apparent only at high substrate concentration. This may be interpreted as an effect on K_{S_2}, and a constant in this case has been chosen to be that concentration of phenylmercuric hydroxide required to make K_{S_2} equal to K_m. The general equation for inhibition by excess of substrate (Dixon & Webb, 1958):

$$v' = \frac{V_{max.}}{1+K_m/S+S/K_{S_2}}, \quad v = \frac{V_{max.}}{1+K_m/S},$$

where v' and v are the observed velocities of the inhibited and uninhibited reactions respectively, S is the substrate concentration and $V_{max.}$ the theoretical maximum velocity for the particular enzyme solution, reduces to $v'/v = \frac{2}{3}$ when $K_{S_2} = K_m = S$. The inhibitor constant is therefore equal to the concentration of phenylmercuric hydroxide causing 33% inhibition when $S = K_m$, and has a value of about 600 μM at pH 4·5 (Fig. 2*b*) and it decreases to 300 μM at pH 5·2 and 200 μM at pH 5·9.

Effect of phenyl β-glucuronide concentration. From the data presented in Fig. 4, K_m is about 900 μM at pH 4·5 and 8 mM at pH 5·9; inhibition by excess of substrate is not normally observed with this enzyme preparation, indicating that the tendency to form an ES_2 complex is weaker. Mercuric and Ag^+ ions behave as with phenolphthalein β-glucuronide at low substrate concentration, but the inhibitory component at high substrate concentration is partly lacking with Ag^+ ion, and with Hg^{2+} ion a decreased inhibition is apparent (Fig. 4*a*). Phenylmercuric hydroxide has a similarly weaker action. *p*-Chloromercuribenzoate inhibits competitively and K_i is about 15 μM, in agreement with the value obtained in the presence of phenolphthalein β-glucuronide at pH 4·5 (derived from the data in Fig. 2*b*) and pH 5·9. With Cu^{2+} ion (Fig. 4*b*), inhibition by excess of substrate does not develop and the residual inhibition appears to be competitive in so far as the inhibition is slightly greater at low substrate concentration. On this basis, K_i for Cu^{2+} ion at pH 5·9 is about 2 mM.

Effect of pH. The pH–activity curves in the presence of phenolphthalein β-glucuronide are

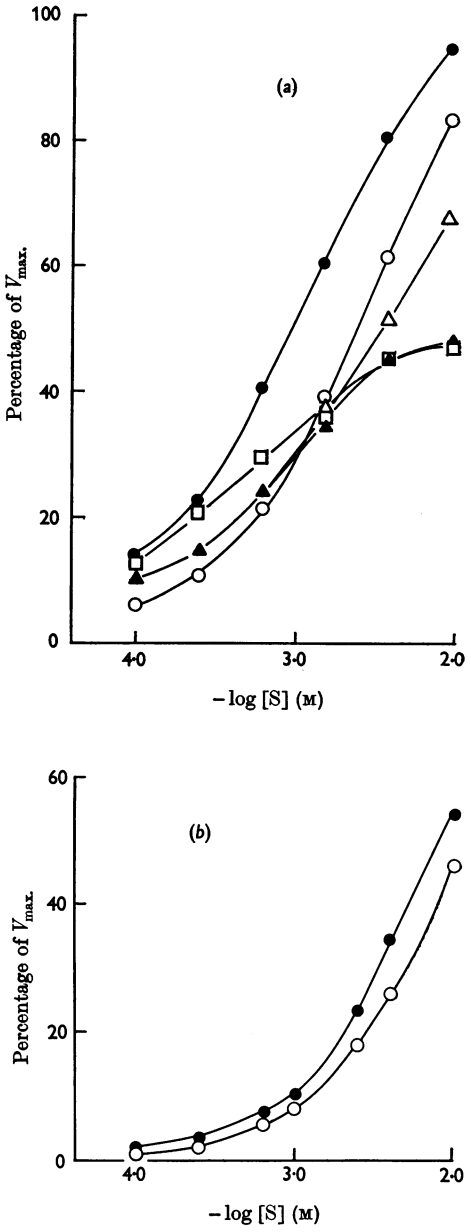

Fig. 4. (*a*) Inhibition of β-glucuronidase by 0·04 mM-$HgCl_2$ (△), 0·16 mM-$AgNO_3$ (▲), 0·04 mM-*p*-chloromercuribenzoate (○) and mM-phenylmercuric hydroxide (□) in 0·05M-acetic acid–NaOH buffer, pH 4·5, as a function of phenyl β-glucuronide concentration. The control series (●) is that for substrate alone ($V_{max.}$ 0·16 mM-phenol). Curves for Hg^{2+} and Ag^+ ions are partially superimposed. (*b*) Inhibition of β-glucuronidase by mM-$CuSO_4$ (○) in 0·05M-acetic acid–NaOH buffer, pH 5·9, as a function of phenyl β-glucuronide concentration. Control series (●) was as described for (*a*) ($V_{max.}$ 0·85 mM-phenol). For other details see the Materials and Methods section.

shown in Fig. 5. The optimum pH is dependent on $V_{max.}$, K_m and K_{S_2}, being that where

$$\frac{V_{max.}}{1+2\sqrt{(K_m/K_{S_2})}}$$

is maximum, with an optimum substrate concentration of $\sqrt{(K_m.K_{S_2})}$. Normally these conditions are fulfilled at pH 4·5 and 0·40–0·63 mM-substrate. The heavy-metal cations shift the optimum pH to the left, indicating that they act more strongly on the alkaline branch of the enzyme activity-determining groups (e.g. Myrbäck, 1926). With Hg^{2+} ion, the more concentrated the inhibitor the greater the shift of the optimum pH. Phenylmercuric hydroxide is also more active on the alkaline side, but, contrary to the expected trend, the optimum pH is more alkaline than normal. The action of this inhibitor on the acid side is stronger simply because the enzyme is already in a region of substrate inhibition. On the alkaline side the enzyme becomes increasingly unsaturated, and consequently phenylmercuric hydroxide is a less effective inhibitor here. For *p*-chloromercuribenzoate, the change in pK_i with pH is shown in Fig. 6 and it is compared with pK_m and the calculated log $V_{max.}$ for phenolphthalein β-glucuronide in 25 mM-acetic acid–25 mM-phosphoric acid–NaOH buffer at each pH. The values were derived from the data presented in Table 2. With this buffer system, values for K_m and K_i were generally higher than those obtained with 0·05 M-acetic acid–NaOH.

Suppression and reversal of inhibitions. Inhibition by Hg^{2+} ions was almost completely suppressed by a variety of reducing agents, e.g. bisulphite, cysteine and thioglycollate (all mM). Chloride, cystine and EDTA (all mM) also suppressed this inhibition and EDTA even increased enzyme activity with phenolphthalein β-glucuronide by about 40% under certain conditions (pH 4·0–4·6, 0·40–0·63 mM-substrate). With phenyl β-glucuronide no such activation occurred and a slight non-competitive inhibition was present throughout the whole substrate concentration range. A possible explanation of the activation is that a mercury–EDTA complex increases K_{S_2}: the maximum activity does not exceed the calculated $V_{max.}$ for the uninhibited enzyme. Reversal of inhibition by Hg^{2+} ions (after 30 min. incubation) with mM-bisulphite or equimolar EDTA was not complete and became less so with increasing incubation time, showing that inactivation also proceeded in the presence of substrate.

Inhibition by Ag^{+} ions was suppressed by mM-chloride or mM-cysteine, unaffected by mM-cystine and potentiated by mM-EDTA (itself inactive). Reversal (with mM-chloride) again depended on the previous incubation time.

Inhibition by Cu^{2+} ions (0·63 mM) was unaffected by mM-cysteine, partially suppressed by mM-cystine and activated beyond the control by mM-EDTA. Unlike the mercury–EDTA mixture, this cupric–EDTA complex also activated the enzyme with respect to phenyl β-glucuronide by about

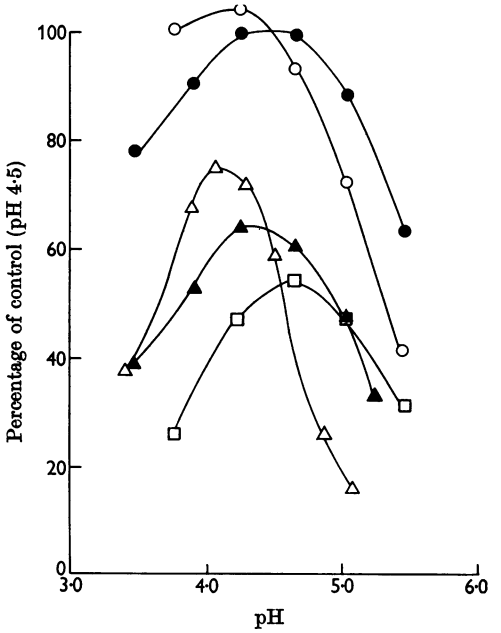

Fig. 5. Inhibition of β-glucuronidase by 0·05 mM-$HgCl_2$ (△), 0·1 mM-$AgNO_3$ (▲), mM-$CuSO_4$ (○) and 0·4 mM-phenylmercuric hydroxide (□) with 0·63 mM-phenolphthalein β-glucuronide and 0·05 M-acetic acid–NaOH buffer as a function of pH. Control series (●) is with substrate alone (100% represents about 30 μM-phenolphthalein). For other details see the Materials and Methods section.

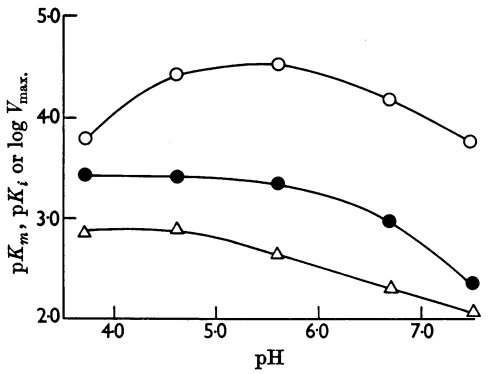

Fig. 6. Effect of pH on K_m (●) and $V_{max.}$ (△) for phenolphthalein β-glucuronide and on K_i (○) for *p*-chloromercuribenzoate. These values are derived from the data presented in Table 2 and refer to a 25 mM-acetic acid–25 mM-phosphoric acid–NaOH buffer system.

Table 2. *Hydrolysis of phenolphthalein β-glucuronide by β-glucuronidase at various* pH *values in the presence of* 0·04 mM-p-*chloromercuribenzoate*

Buffer (25 mM-acetic acid–25 mM-phosphoric acid–NaOH at the appropriate pH), substrate and inhibitor were brought to 38° and the reaction was started by addition of enzyme in 0·01 % albumin. Each initial substrate concentration (S_o) produced the indicated concentration (μM) of phenolphthalein in the absence (–) and presence (+) of *p*-chloromercuribenzoate after incubation for 60 min. with enzyme at 38°. Other details are given in the Materials and Methods section.

	pH 3·7		pH 4·6		pH 5·6		pH 6·7		pH 7·5	
S_o (μM)	–	+	–	+	–	+	–	+	–	+
40	4·4	3·6	4·6	2·7	4·8	2·1	2·9	1·8	1·8	1·6
63	6·4	5·2	6·9	3·5	6·9	3·1	3·9	2·7	2·8	2·3
100	9·4	7·8	10·2	5·9	10·3	4·8	6·5	4·1	4·2	3·5
160	14·2	12·4	14·4	8·7	15·0	7·1	9·5	6·3	6·8	5·4
250	18·4	16·3	20·0	13·4	20·8	11·1	13·5	9·1	9·8	8·0
400	21·7	20·0	23·6	18·5	27·0	16·2	19·0	13·0	16·0	13·0
630	22·0	19·5	24·6	19·7	32·2	21·8	27·1	21·6	24·4	19·5
1000	19·1	15·3	22·7	19·0	34·6	26·4	36·6	33·2	35·8	29·2
1600	6·3	4·2	19·0	17·1	34·6	27·8	45·5	38·0	52·5	43·0
2500	3·4	1·8	14·6	9·1	31·9	26·6	52·0	44·5	69·0	59·4
Enzyme (μg. of protein)	0·08		0·08		0·16		0·49		1·95	

50 %. It appears to be non-competitive. Reversal with mM-EDTA (after 30 min. incubation) was again not complete.

Phenylmercuric hydroxide and *p*-chloromercuribenzoate inhibitions were completely suppressed and reversed by mM-cysteine, unaffected by mM-cystine and, with mM-EDTA, *p*-chloromercuribenzoate was unaffected, but phenylmercuric hydroxide inhibition was partially suppressed.

Effect of copper in the presence of reducing agents

Ascorbic acid. At pH 4·5 in the presence of mM-ascorbate or dihydroxymaleate, only trace amounts of Cu^{2+} ions were required to inactivate β-glucuronidase completely. This is shown in Fig. 7, where, even with specially purified reagents, inactivation in the absence of added copper is evidenced. A non-enzymic hydrolysis of substrate can occur under these conditions (see Materials and Methods section) and must either be allowed for by including the appropriate substrate–ascorbate blanks (with and without Cu^{2+} ions) in studying the effect in the presence of substrate, or eliminated by adopting a procedure such as that described in Fig. 7, where residual enzyme activity is measured. Substrate does not affect the destruction of the enzyme, so both procedures lead to the same final result. EDTA arrested the inactivation and albumin gave protection in the absence of added copper, indicating that Cu^{2+} ions were involved in the reaction. Oxygen was not found to be an influencing factor. Of the ions Cu^{2+}, Au^{3+}, Ag^{+}, Hg^{2+}, Cr^{3+}, Zn^{2+}, Pb^{2+} and Fe^{3+} (each μM) tested in the above-mentioned type of experiment, Cu^{2+} ions had the most pronounced action, Au^{3+} ions the next and Fe^{3+} ions the least.

A solution of CuCl (0·4 mM) produced 60 % inactivation when added to the enzyme in 0·05 M-acetic acid–NaOH buffer, pH 4·5. The initial concentration of Cu^{+} ions under these conditions was only half that of the added CuCl, and was immeasurably low (less than μM) a few seconds after the addition. Inactivation by Cu^{+} ions must therefore have been extremely rapid. It is likely that the role of ascorbate lies in its capacity to reduce cupric to cuprous copper (dehydroascorbate being demonstrably ineffective) and to maintain it as such. The amount of Cu^{+} ion required to inactivate the enzyme completely is certainly less than 0·1 μM, but the reaction is also time-dependent and so the minimum copper requirement is not known. Subsequent addition of cysteine, cystine or EDTA (all mM) did not reverse this inactivation.

Bisulphite. Above pH 5 only, 0·01 mM-Cu^{2+} ions in the presence of mM-bisulphite catalysed a progressive inactivation of β-glucuronidase. This differed from the ascorbate system in that it was rather less active (Fig. 8) and was suppressed in the presence of substrate (competitive inactivation). The process was prevented and arrested by EDTA, but was not reversed by this reagent or by mM-cysteine or mM-cystine. Though bisulphite alone has no effect on the enzyme, there is usually sufficient endogenous copper under assay conditions to catalyse extensive inactivation.

Thioglycollate. At pH 4·5, mM-thioglycollate and 0·1 mM-Cu^{2+} ions produced an apparent non-competitive inhibition (about 40 %), similar to that obtainable with 0·05 mM-ascorbate and 0·01 mM-Cu^{2+} ions, which suggests that an inactivation is also involved here.

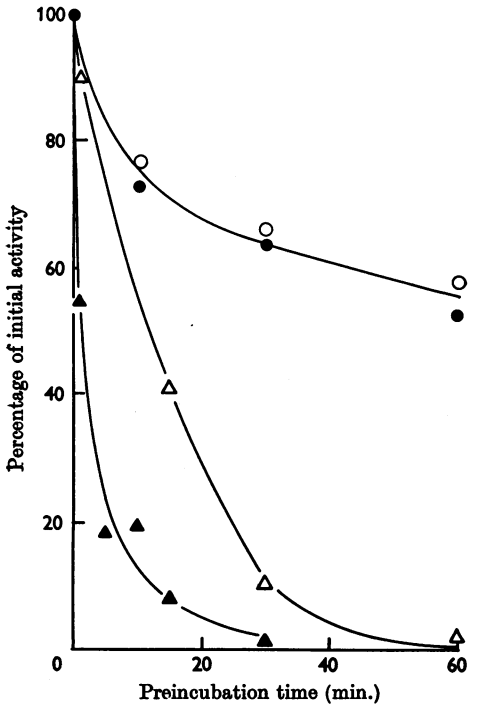

Fig. 7. Inactivation of β-glucuronidase in the absence of albumin (about 10 μg. of protein/ml.) by Cu^{2+} ions and ascorbate in 0·05M-acetic acid–NaOH buffer, pH 4·5, at 38°. The enzyme was assayed by introducing samples of the mixtures, at intervals, into 0·63 mM-phenolphthalein β-glucuronide, 0·01 mM-EDTA and 0·01% of albumin in 0·05M-acetic acid–NaOH buffer, pH 4·5, and incubating for 20 min. at 38°. The curves refer to enzyme alone (●) and enzyme in the presence of mM-ascorbate (△), mM-ascorbate plus 0·01 mM-EDTA (○) and mM-ascorbate plus added 0·1 μM-$CuSO_4$ (▲). Initial activity per sample was about 200 glucuronidase units.

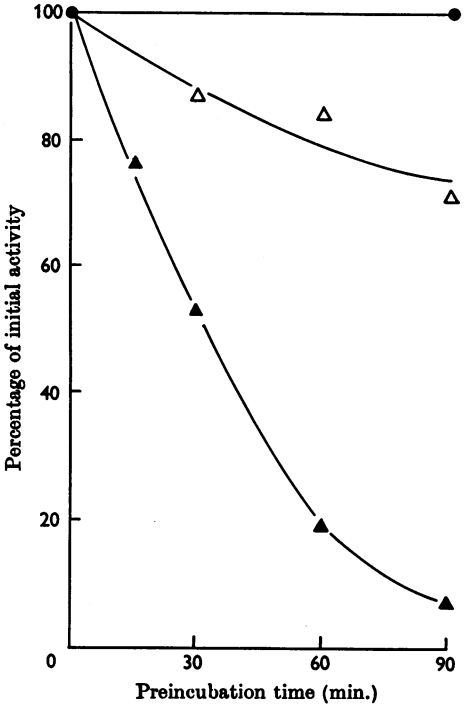

Fig. 8. Inactivation of β-glucuronidase in the presence of 0·01% of albumin by Cu^{2+} ions and bisulphite in 0·05M-acetic acid–NaOH buffer, pH 5·2, at 38°, before addition of 0·63 mM-phenolphthalein β-glucuronide, pH 5·2. This arrested the inactivation and also measured the residual enzyme activity (initially 32 μM-phenolphthalein). Curves refer to enzyme–albumin alone (●), enzyme–albumin plus mM-bisulphite (△) and enzyme–albumin plus mM-bisulphite and 0·01 mM-$CuSO_4$ (▲). For other details see the Materials and Methods section: 'General assay procedure (*b*).'

Cysteine. Under certain conditions, cysteine or thiosulphate will cause copper to inactivate β-glucuronidase, but their capacity to remove copper from the system limits their efficacy in this respect. Thus a mixture of 0·2 mM-cysteine and mM-Cu^{2+} ions, pH 4·5, produced 35% inactivation, whereas mM-cysteine and 0·1 mM-Cu^{2+} ions had no effect.

DISCUSSION

The actions of Hg^{2+} and Ag^{+} ions on β-glucuronidase are qualitatively similar to one another; both cause a certain amount of inactivation in the presence or absence of substrate and both have non-linear velocity curves, which were unaffected by preincubation of inhibitor with enzyme or substrate alone. Mercury and silver must therefore react with an enzyme–substrate complex, but it is not possible to say whether this is a 'combined' inhibition:

$$E+S \rightleftharpoons ES; \quad ES+I \rightleftharpoons ESI,$$

or a form of non-competitive inhibition:

$$E+S \rightleftharpoons ES; \quad ES+I \rightleftharpoons ESI \rightleftharpoons EI+S,$$

or even some other type of inhibition. Velocity curves with phenyl β-glucuronide show a decreasing linearity but an increasing initial slope with increasing substrate concentration, which suggests a twofold effect: one involving a ternary complex (as shown above) and the other being a straightforward competitive action. With phenolphthalein β-glucuronide, a third component acting on K_{S_2} should be added to the inhibition since here the initial slope of the velocity curves decreases at high substrate concentration. Because the velocity curves are not linear, it follows that the data relating to the various activity curves in the

presence of Hg^{2+} and Ag^{+} ions do not represent the equilibrium values and hence no equilibrium constants can be derived. With Cu^{2+} ions, the situation is different: the velocity curve is basically linear, the system is in equilibrium and may be analysed. The action of the organic mercurials on the enzyme is less complex than that of the heavy-metal cations; there is no inactivation and equilibrium is reached very quickly.

p-Chloromercuribenzoate has a relatively strong affinity for β-glucuronidase. K_i in acetate buffer is constant between pH 4·5 and 6 at about 15 μM, and in acetate–phosphate buffer reaches a minimum value of about 35 μM at pH 5·5. The measured affinity between enzyme and inhibitor represents a difference in affinities between two systems, one involving enzyme and inhibitor and the other involving inhibitor and environment. For this reason variations in heavy-metal inhibitions with pH and the nature of the buffering medium must be interpreted with caution. The situation is further complicated by a possibility of interaction between the enzyme and anions, other than the substrate, of necessity present in the medium. For instance, the data in Table 2 show that phosphate increases K_m without materially influencing $V_{max.}$ and hence is behaving as a weak competitive inhibitor. The action of citrate is very similar, though strongly pH-dependent (Mills, Paul & Smith, 1953). Conflicting reports in the literature on the status of various other organic acids as weak inhibitors of β-glucuronidase (see Levvy & Marsh, 1959) may be due to the different pH values and relative substrate concentrations employed by different workers; Mills *et al.* (1953) found oxalate, malonate and succinate (each 10 mM) to be inhibitory at pH 3·4, whereas at pH 5·2 Oshima (1936) regarded these compounds (20 mM) as non-inhibitory. There is a strong suggestion that this phenomenon reflects a competition between the carboxyl group of the glucuronide and an acidic group of the anion (and hence a particular ionic species) for the enzyme. As *p*-chloromercuribenzoate reacts with anions (Boyer, 1954) and also competes for the active centre, it is not surprising that K_i should vary with the nature of the buffer.

Inhibition by *p*-chloromercuribenzoate is reversed by cysteine, but not by cystine or EDTA, and this behaviour is consistent with reaction between the mercurial and a SH group in the enzyme (Boyer, 1959). On the other hand, phenylmercuric hydroxide [which is also known to be a SH reagent (Cecil & McPhee, 1959)] has no resemblance in its order of affinity for the enzyme, in its mode of action or in its change of affinity with pH. *p*-Chloromercuribenzoate increases K_m, phenylmercuric hydroxide does not; the latter decreases K_{S_2}, the former does not, though the possibility that higher concentrations of *p*-chloromercuribenzoate might affect K_{S_2} cannot be ruled out. Cupric copper, with phenolphthalein β-glucuronide as substrate, exhibits the combined actions of the organic mercurials. With phenyl β-glucuronide it only affects K_m because K_{S_2} is too large to be observable under normal experimental conditions (in the presence of mM-phenylmercuric hydroxide and 0·16 mM-Ag^{+} ions, however, there is a suggestion of inhibition by excess of substrate).

To sum up, it would appear that the inhibitory actions of these heavy-metal ions can be explained on the basis of three separate effects: (i) a competition between substrate and inhibitor, observed with *p*-chloromercuribenzoate, Hg^{2+}, Ag^{+} and Cu^{2+} ions, which suggests that a SH group is located in the active centre of the enzyme; (ii) a tendency to promote formation of an inactive ES_2 complex, observed with phenylmercuric hydroxide, Hg^{2+}, Ag^{+} and Cu^{2+} ions; (iii) a combination with ES but not with E or S alone, observed with Hg^{2+} and Ag^{+} ions.

Above pH 5, Cu^{2+} ions catalyse a substrate-protected inactivation of β-glucuronidase by bisulphite. This action of bisulphite (or possibly sulphite) is thought to involve a disulphide group in the enzyme (see Cecil & McPhee, 1959; Cecil & Loening, 1960):

$$RS{\cdot}SR + SO_3^{2-} \rightleftharpoons RS{\cdot}SO_3^{-} + RS^{-}.$$

This suggests that this grouping also is present at the active centre. The participation of Cu^{2+} ions in the reaction is an interesting phenomenon that may possibly be relevant to other cases in which disulphide cleavage by bisulphite is found to occur.

Cuprous copper is the most toxic of the heavy-metal ions investigated. With ascorbate as a source of Cu^{+} ions, only 0·1 μM-copper is required for complete inactivation. The reaction is uninfluenced by the presence of substrate and it can occur below pH 5 (though inactivation is more pronounced at pH 5·9). An analogous role of ascorbate in the inhibition of urease by copper has been described by Mapson (1946) and a similar mechanism has been proposed by Rowe & Weill (1959) for β-amylase. In both these examples, the cuprous copper-treated enzyme was reactivated by cysteine, but there is no evidence that a reactivation can occur with β-glucuronidase.

SUMMARY

1. The effects of copper, silver, mercury, *p*-chloromercuribenzoate and phenylmercuric hydroxide on purified β-glucuronidase from female-rat preputial gland have been investigated. Experiments in which inhibitor concentration, substrate, substrate concentration, pH and time have been varied are described.

2. Mercuric chloride and silver nitrate have a qualitatively similar inhibitory action on the enzyme. This appears primarily to be competitive, but an effect on K_{S_2}, the dissociation constant of the inactive ES_2 enzyme–substrate complex, can also be distinguished, thereby increasing substrate inhibition. A third effect develops only in the presence of substrate, increasing the overall inhibition. Phenylmercuric hydroxide exhibits a novel form of inhibition in that it only decreases K_{S_2}. *p*-Chloromercuribenzoate is a purely competitive inhibitor and appears not to affect K_{S_2}. Cupric sulphate probably inhibits competitively and also decreases K_{S_2}, so combining the two independent actions of the organic mercurials.

3. The inactivating action of copper is potentiated by various reducing agents. Ascorbate is shown to act by reducing cupric copper to the much more toxic cuprous form. In the presence of bisulphite, copper catalyses a substrate-protected decomposition of the enzyme.

4. It is concluded that both SH and SS groups are involved in the active centre of β-glucuronidase.

5. Cuprous ion is shown to cause a non-enzymic hydrolysis of several aryl glucosides, including phenolphthalein β-glucuronide.

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Polysaccharides of *Chlorella pyrenoidosa*

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Although the polysaccharides of the green algae (Chlorophyceae) have not been investigated as extensively as those of the red and brown algae, the few that have been studied seem to show certain structural characteristics and are different from those of the other classes of algae. In most of the work on the water-soluble polysaccharides of the green algae criteria of homogeneity have not been established for the materials isolated and therefore it is difficult to assess the significance of the structural work carried out on these products. However, from the work on the polysaccharides