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CHANGES IN THE pH-ACTIVITY CURVE OF β-D-GLUCURONIDASE IN THE LIVER AND SPLEEN OF MICE AFTER INTRAPERITONEAL ADMINISTRATION OF VARIOUS MACROMOLECULAR SUBSTANCES

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Abstract—The increase in the activity of β-glucuronidase as a result of storage of macromolecular substances in the liver and spleen of mice was studied. Dextran and polymers of vinylpyrrolidone were injected intraperitoneally into mice of the O_{20} strain and Swiss mice. The activity of β-glucuronidase was investigated in liver and spleen homogenates between pH 3·2 and pH 7·0. It was shown that with the strains used, there were present in the pH–activity curves of β-glucuronidase of liver and spleen homogenates two distinct peaks between pH 4·3 and 5·1 and probably a small one at pH 3·5. As a result of the incorporation of the macromolecular substances, the activity of the entire enzyme complex rises. The increase in activity is not, however, equally large for the entire pH range studied. In liver homogenates, after administration of the macromolecular substances, the activity of the enzyme complex showed relatively the largest increase between pH 3·2 and 3·5.

IN A previous investigation[1] solutions of the macromolecular substances dextran, polyvinylpyrrolidone and a suspension of carbon were injected into the peritoneal cavity of mice. The acid phosphatase and β-glucuronidase activities in the liver and spleen were investigated histochemically and biochemically, and the localization of the macromolecular material stored in these organs was investigated histochemically. The histochemical results showed that the macromolecular substances were stored in the cytoplasm of the Kupffer cells. Dextran was also stored in the cytoplasm of the parenchymal cells. In the spleen the macromolecular substances were stored exclusively in the cytoplasm of the reticulum cells.

In the livers of the control animals, activity of the two enzymes was found almost exclusively in the Kupffer cells. In the livers of injected animals, activity of the two enzymes was seen in both the Kupffer and the parenchymal cells. In the spleen only the reticulum cells surrounding the follicles showed acid phosphatase and β-glucuronidase activity.

The biochemical results showed that the total activity of acid phosphatase and β-glucuronidase in the liver and spleen of mice injected with the macromolecular substances was significantly increased compared with control animals. The activity of the enzymes acid phosphatase and β-glucuronidase were determined at pH 4·8 and pH 4·5, respectively. Since the activity of both enzymes is strongly dependent on pH, it was of importance to study the variation in enzyme activity at other pH-values of the incubation medium after intraperitoneal administration of macromolecular

substances. Therefore, in another investigation[2] the pH–activity curves of acid phosphatase were studied in the liver and spleen of injected and control mice. It was shown that in both liver and spleen extracts three different non-specific acid phosphatases were present, characterized by different pH optima. As a result of the incorporation of the macromolecular substances, the activity of the entire enzyme complex increased. This increase in activity, however, was not equally large for all the acid phosphatase enzymes. In the liver, after administration of dextran the activity of the enzyme with an optimum at pH 5·8 showed relatively the largest increase.

In the investigation reported below, the pH–activity curves of β-glucuronidase were studied in the liver and spleen of control mice and injected mice of the strain O_{20} (Amsterdam) and Swiss mice. The investigation demonstrated that as a result of the storage of macromolecular substances the activity of the entire enzyme complex increased in both organs at pH values between 3·2 and 7·0, but unlike that for the enzyme acid phosphatase, the shape of the pH–activity curve shows little modification. In addition, the shape of the β-glucuronidase pH–activity curves is dependent on the strain of mouse used.

METHOD

Mice of the O_{20} strain (Amsterdam) and Swiss mice between 6 and 8 months old were used. After intraperitoneal administration of macromolecular substances, the activity of β-glucuronidase was determined in liver and spleen homogenates.

Macromolecular substances

1. Dextran, with an average molecular weight of 200,000. The dextran was obtained from Poviet & Co, Amsterdam. From the dextran a 6% sterile solution in 0·9% NaCl was prepared.

2. Polyvinylpyrrolidone, a polymer of vinylpyrrolidone with an average molecular weight of 640,000 (Bayer, Leverkusen). A 6% sterile solution in 0·9% NaCl was prepared.

3. Polyvinylpyrrolidone, with an average molecular weight of 50,000 (Bayer, Leverkusen). A 6% sterile solution in 0·9% NaCl was prepared.

Injection scheme

For the O_{20} mice, five groups of animals were examined, each group consisting of five animals. The animals were killed on the tenth day after the first injection. The activity of β-glucuronidase was determined in homogenates of the livers and spleens. One control group (I) received no treatment. A second control group (II) was given daily intraperitoneal injections of 1 ml of 0·9% NaCl solution for 9 days and fasted for 18 hr before being killed on the tenth day. Group III was given daily intraperitoneal injections of 1 ml of dextran solution for 9 days and fasted for 18 hr before being killed on the tenth day. Group IV was given daily interperitoneal injections of 1 ml of polyvinylpyrrolidone solution (640,000) for 9 days and fasted for 18 hr before being killed on the tenth day. Group V was given daily intraperitoneal injections of 1 ml of polyvinylpyrrolidone solution (50,000) for 9 days and fasted for 18 hr before being killed on the tenth day.

For the Swiss mice four groups of animals were examined (I, II, III and V), each group consisting of from four to five animals.

Preparations of homogenates

Small quantities of tissue (200 mg) taken from various parts of the liver and from the whole spleen were weighed rapidly on a torsion balance. The tissue was homogenized with double-distilled water for 3 min in a ground-glass Potter–Elvejhem homogenizer. After homogenizing, more distilled water was added to bring the tissue concentration to 100 mg tissue per 20 ml water. To ten parts of this solution was added one part of a 1% solution of Triton-X-100 to achieve maximal solubilization and availability of the enzyme for interaction with its substrate.[3] The homogenates to which Triton-X-100 had been added were allowed to stand for 60 min at 0 °C.

β-D-Glucuronidase activity determinations

The activity of the enzyme β-glucuronidase in the homogenate was determined using phenolphthalein-β-D-monoglucuronide as a chromogenic substrate, the liberated phenolphthalein being determined colorimetrically. The substrate phenolphthalein β-D-monoglucuronide was prepared according to Fishman *et al.*[4] The incubations were carried out as described by Talalay[5] at 37 °C, except that the assays were done with smaller quantities. After incubation, the enzyme reactions were terminated by addition of a glycine–NaOH buffer solution with a pH of 10·5. Because the molar extinction of phenolphthalein is to a high degree dependent upon the pH of the *milieu* and in this study buffer solutions of various pH values were used, after incubation the enzyme reactions were terminated by the four-fold quantity of glycine buffer according to Talalay. In spite of the relatively large excess of glycine buffer, small differences in pH were still present after addition of the buffer solution. Hence, for the calculation of the activities, a separate phenolphthalein straight line was constructed for every given pH value of the incubation medium. Each determination was made in triplicate with a single control. The relative standard deviation of the triple determinations was smaller than 3 per cent.

Buffer solutions

The activity of β-glucuronidase is strongly dependent on the type of buffer solution present in the incubation flasks.[6, 7] For this reason in the present study use was made of only one type of buffer solution, namely 0·1 M buffer solutions of acetic acid and sodium acetate. The pH values of the incubation mixtures obtained with the ten buffer solutions used were 3·2, 3·5, 3·9, 4·3, 4·6, 4·8, 5·1, 5·4, 6·0 and 7·0, respectively. Tests showed that the differences in the pH values of the incubation solutions before and after incubation were never greater than 0·05 pH units.

RESULTS

Incubation time

Experiments in which the quantity of phenolphthalein liberated by the enzyme was determined after various incubation intervals showed that the stability of the enzyme is dependent on the pH of the incubation solutions. In these experiments an investigation was made of the activity of β-glucuronidase in liver and spleen homogenates of control animals and of animals injected with macromolecular substances. It was shown that the stability of the enzyme does not significantly change under the influence of intraperitoneal injections of the various macromolecular substances used. The

stability was lowest in the incubation solutions with the pH values 3·2 and 3·5. An incubation period of 60 min was used. With this incubation period any inactivation of the enzyme in the incubation flasks with the pH value of 3·2 would in any case be less than 10 per cent and with the pH value of 3·5 less than 8 per cent.

pH activity curves

Figs. 1–8 give the pH–activity curves. The activity of β-glucuronidase is plotted against the pH. The activity is expressed as the calculated quantity of phenolphthalein in μg liberated by the entire organ during an incubation time of 1 hr, and the resultant value divided by the weight of the mouse. This method provides the most easily comparable results.[1, 8]

Figs. 1 and 2 show the activity of β-glucuronidase in the liver and spleen of mice of the O_{20} strain injected with 0·9 % NaCl in water (Group II). Since the β-glucuronidase activity in the liver and spleen of the untreated animals (Group I) gives practically the same results as those found for Group II, no figures are given for Group I.

Figs. 3 and 4 show the activity of β-glucuronidase in the liver and spleen of mice of the O_{20} strain injected with dextran (Group III).

Since the activity of the enzyme in the liver and spleen of the animals injected with polyvinylpyrrolidone (Groups IV and V) gave almost the same results as those found for Group III, no figures are given.

Figs. 5 and 6 show the activity of β-glucuronidase in the liver and spleen of untreated Swiss mice. Since the activity of β-glucuronidase in the liver and spleen of animals injected with 0·9 % NaCl in water (Group II) gave practically the same results as those found for Group I, no figures are given.

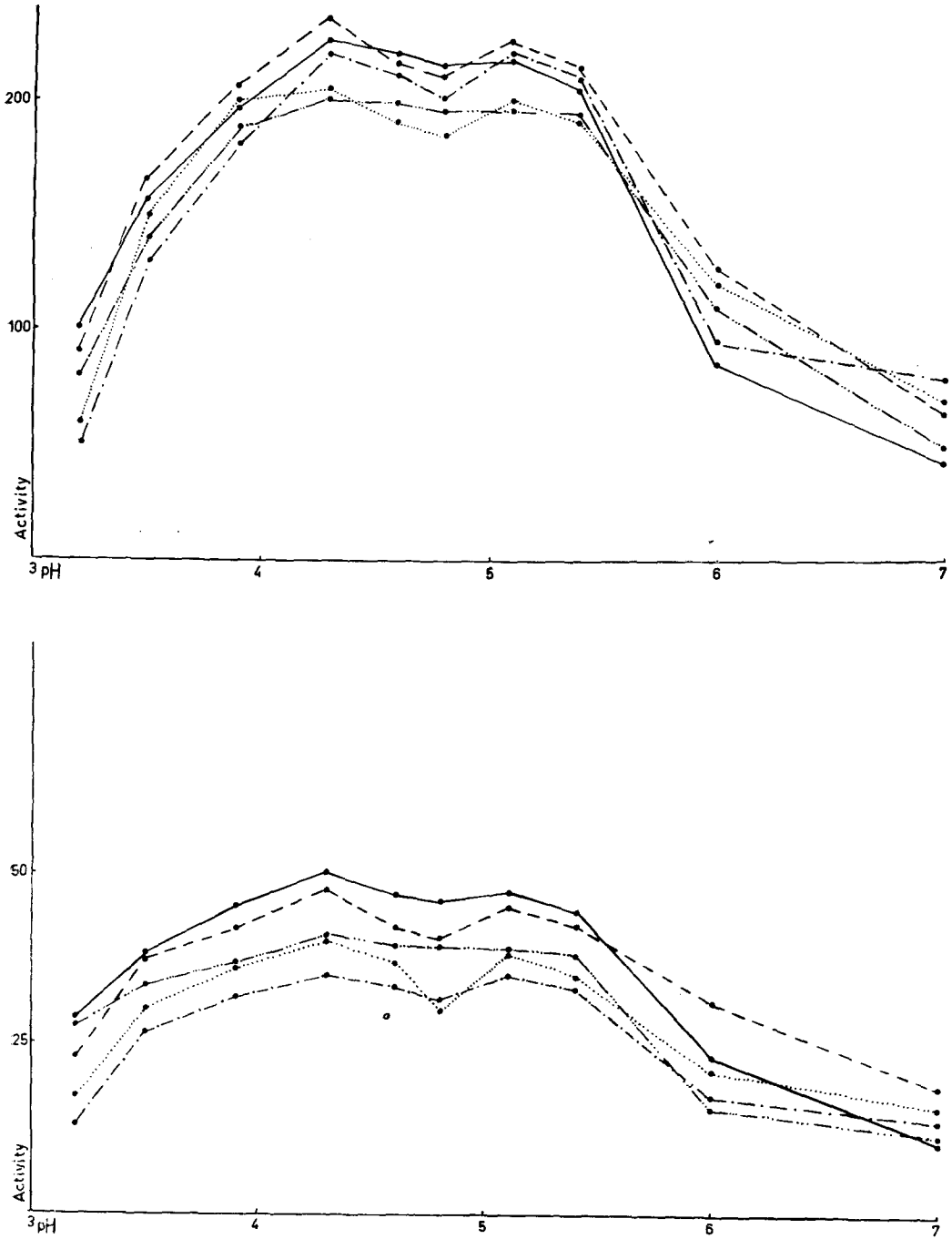
Figs. 7 and 8 show the activity of β-glucuronidase in the liver and spleen of Swiss mice injected with dextran (Group III). Since the activity of β-glucuronidase in the liver and spleen of animals injected with polyvinylpyrrolidone (50,000) (Group V) gave practically the same results as those found for Group III, no figures are given.

DISCUSSION

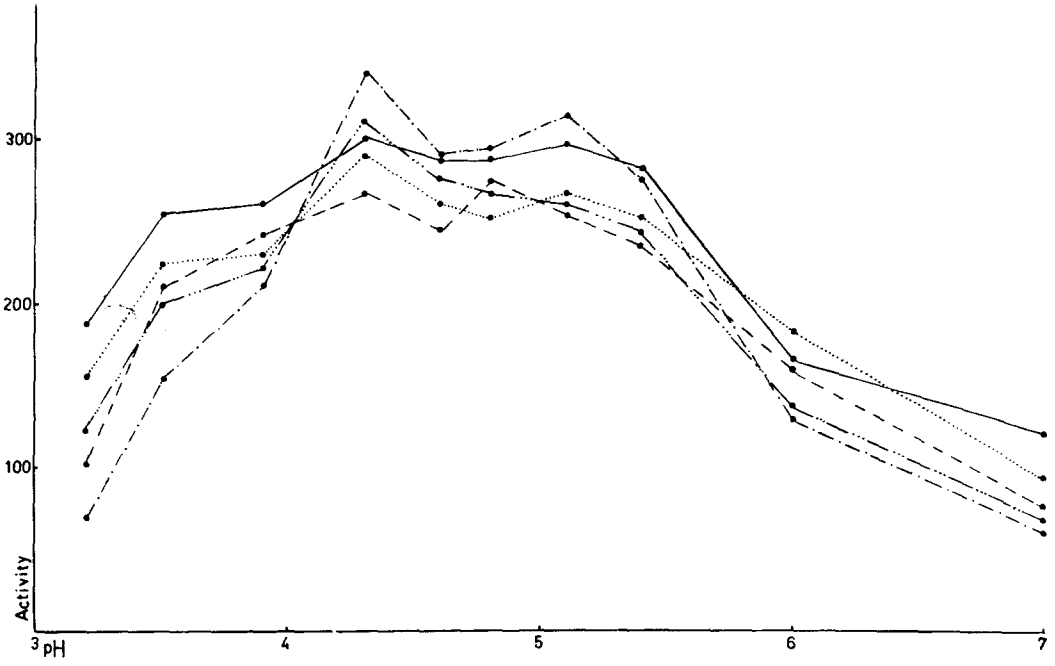
The pH–activity curves obtained with the O_{20} mice were characterized by two peaks. For all groups of animals and for both organs, the peaks were located at about the same pH values, i.e. pH 4·3 and 5·1. In a few animals the peak at pH 5·1 is shifted to pH 4·8, and this shift is found for both the liver and the spleen homogenates in most of these cases. The systematic errors in the activity determinations are such that the peaks are statistically significant.

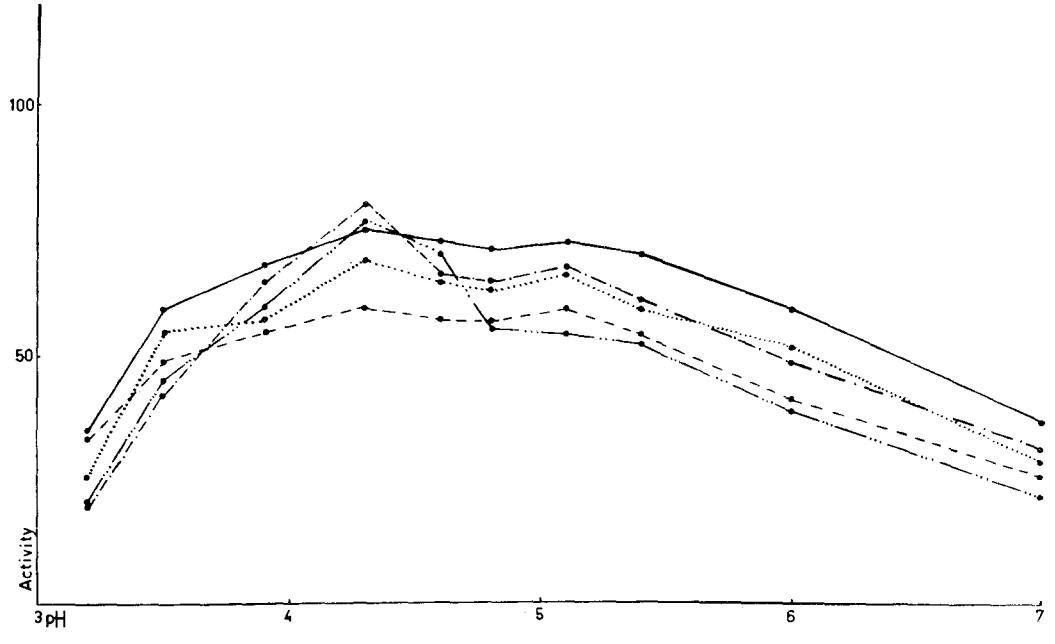
The pH–activity curves of the control mice (Group I) which received no treatment are identical with the curves given in Figs. 1 and 2. It follows from this that in agreement with previous observations,[1] the injections with physiological saline produce no effect on the activity of the enzyme.

After injection of the O_{20} mice with solutions of macromolecular substances, the enzyme activity increased in the liver and spleen homogenates over the entire pH range under investigation. This increase in activity is not statistically significant in

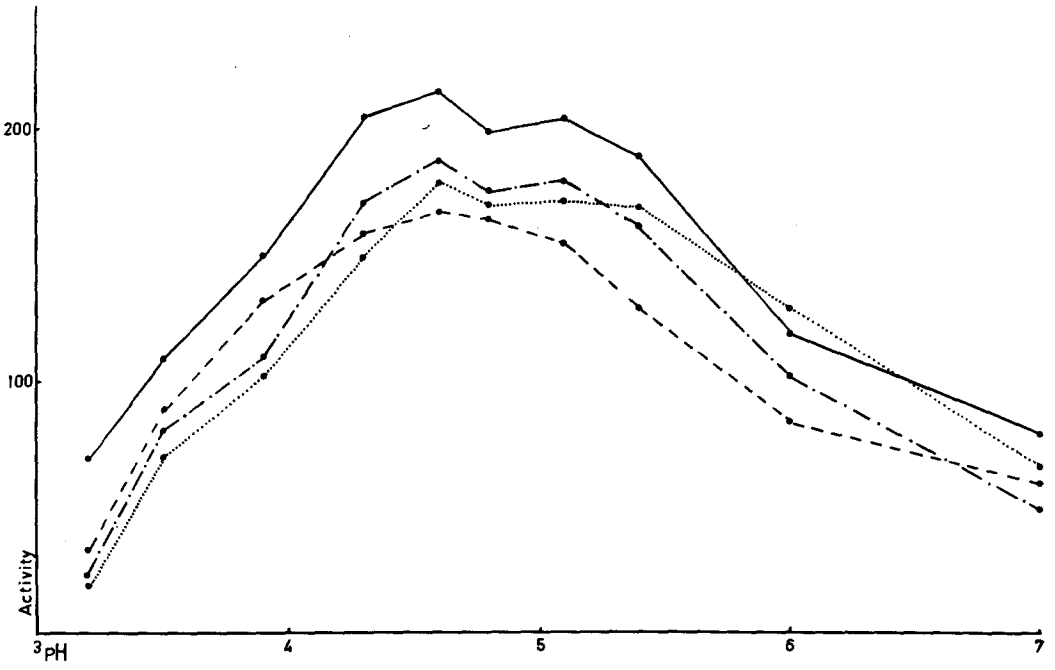


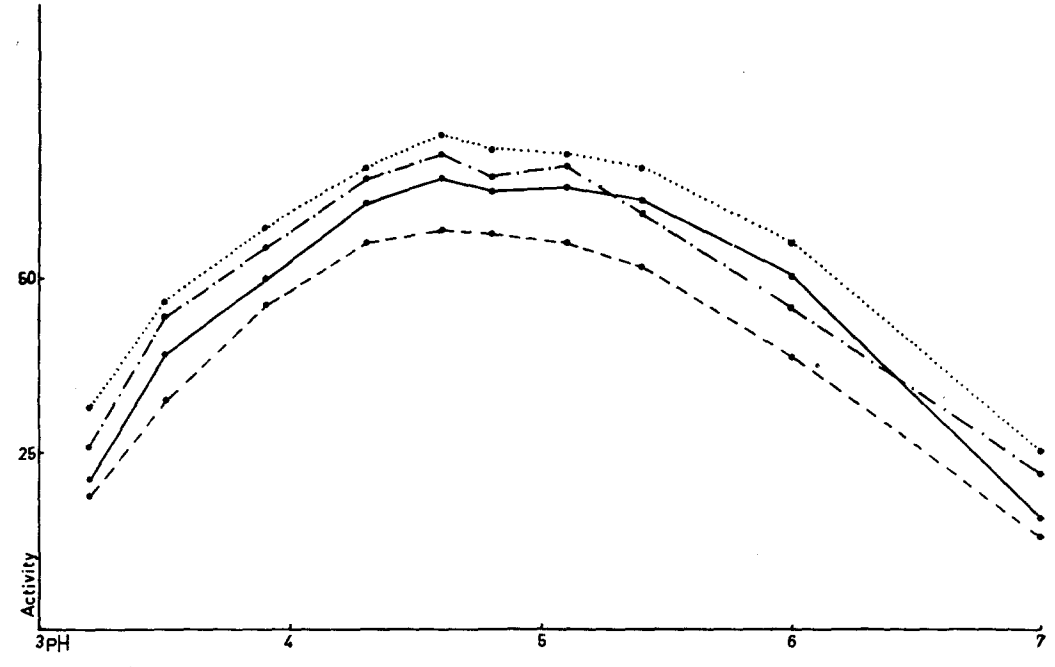
FIGS. 1 and 2. The activity of β-glucuronidase in the liver and spleen of five mice of the O_{20} strain given 0·9% NaCl in water (Group II). Activity is μg phenolphthalein (organ)/g (mouse)/hr. The activity of the enzyme in the two organs of the five mice is shown by———, — — —, —·—·—, —··—··— and .....



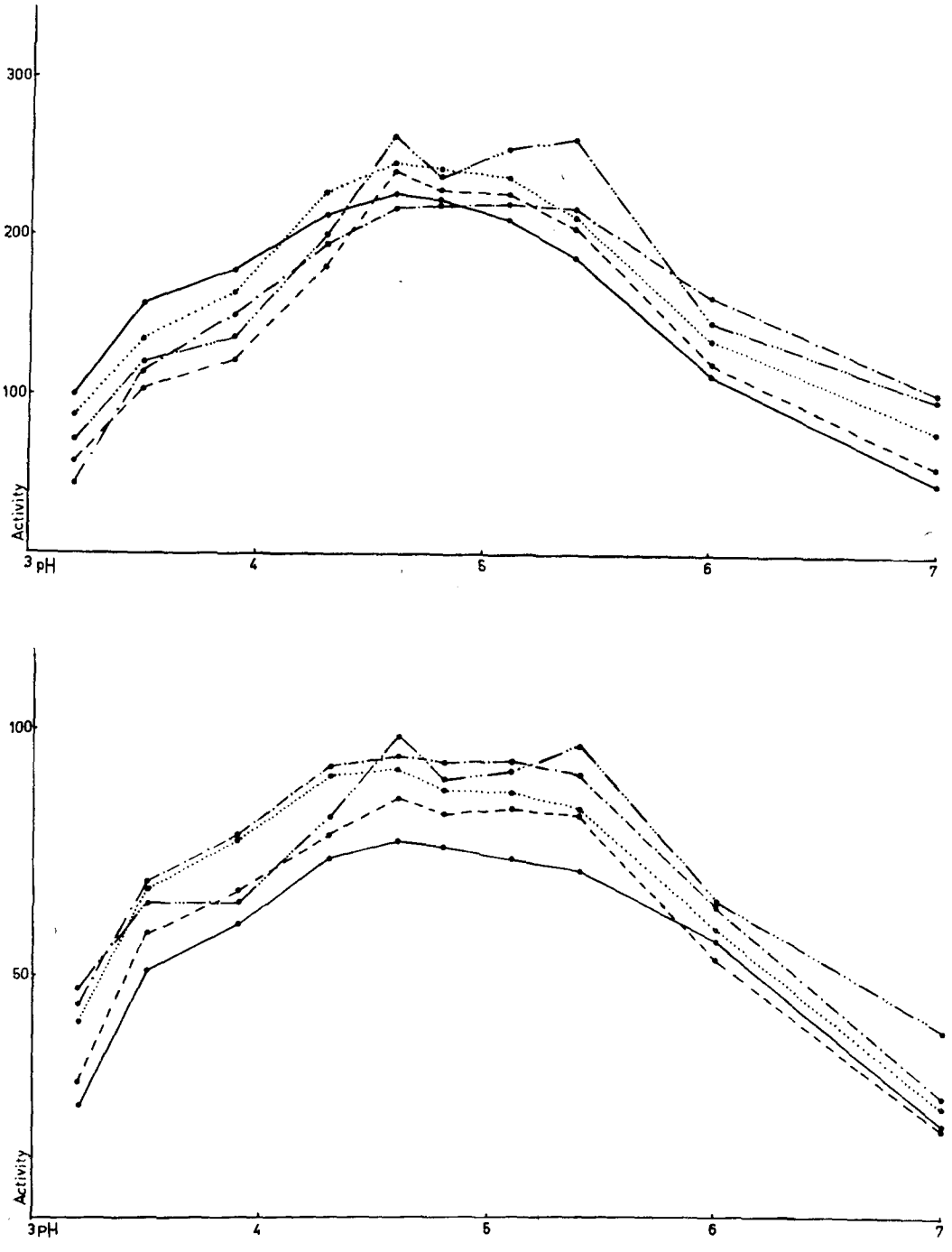


FIGS. 3 and 4. The activity of β-glucuronidase in the liver and spleen of five mice of the O_{20} strain injected with dextran (Group III).





FIGS. 5 and 6. The activity of β-glucuronidase in the liver and spleen of four Swiss mice which received no injections (Group I).



FIGS. 7 and 8. The activity of β-glucuronidase in the liver and spleen of five Swiss mice injected with dextran (Group III).

the liver homogenates for the pH range from pH 6·0 to pH 7·0, however ($P > 0{\cdot}05$). Calculation shows that, in distinction to the spleen homogenates, the relative increase* in activity in the liver homogenates is not the same for all the pH values. The relative increase in the pH range from 3·2 to 3·5 is for the liver homogenates significantly greater ($P \leqslant 0{\cdot}05$) than in the pH range from 6·0 to 7·0. The relative increase of the activities in the liver and spleen homogenates at the various pH values of the incubation media are shown in Tables 1 and 2.

TABLE 1. β-GLUCURONIDASE, LIVER O_{20} MICE

(Activity relative to controls of the enzyme in liver homogenates of O_{20} mice at various pH values of the incubation medium.)

Group	pH									
	3·2	3·5	3·9	4·3	4·6	4·8	5·1	5·4	6·0	7·0
I and II controls	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)
III dextran, m.w. = 200,000	1·7	1·5	1·3	1·4	1·4	1·4	1·4	1·4	1·3	1·3
IV PVP, m.w. = 640,000	1·7	1·7	1·4	1·4	1·4	1·7	1·6	1·6	1·2	1·3
V PVP, m.w. = 50,000	1·7	1·5	1·3	1·4	1·4	1·5	1·7	1·2	1·1	1·1
mean	1·7	1·6	1·3	1·4	1·4	1·5	1·6	1·4	1·2	1·2

TABLE 2. β-GLUCURONIDASE, SPLEEN, O_{20} MICE

(Activity relative to controls of the enzyme in spleen homogenates of O_{20} mice at various pH values of the incubation medium.)

Group	pH									
	3·2	3·5	3·9	4·3	4·6	4·8	5·1	5·4	6·0	7·0
I and II controls	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)
III dextran, m.w. = 200,000	1·4	1·5	1·6	1·7	1·9	1·9	1·7	1·6	1·8	1·7
IV PVP, m.w. = 640,000	1·5	1·6	1·5	1·5	1·7	1·7	1·5	1·4	1·6	1·6
V PVP, m.w. = 50,000	1·6	1·6	1·6	1·6	1·8	1·9	1·6	1·6	1·8	1·8
mean	1·5	1·6	1·6	1·6	1·8	1·8	1·6	1·5	1·7	1·7

The pH–activity curves show that the activity may be due to two different enzymes with optimal pH values at 4·3 and 5·1. Although in the control animals no distinct peak is observed at pH 3·5, the pH–activity curves of the liver homogenates of O_{20} animals injected with solution of macromolecular substances give the impression that there may be still a third enzyme with an optimal activity at approximately pH 3·5, present in the homogenates.

The results obtained with the Swiss mice broadly resemble those for the O_{20} mice. Most of the pH–activity curves were here too characterized by two peaks. The first peak is, compared with that of the O_{20} mice, shifted slightly to the right and lies in the neighbourhood of pH 4·6. The second peak shows no shift and lies in the neighbourhood of pH 5·1. It is remarkable, however, that the pH–activity curves of a few liver

* By the relative increase of the activity at a given pH value of the incubation medium is understood the average activity at this pH in the five liver or spleen homogenates of a group of animals injected with a given macromolecular substance divided by the average activity of the same enzyme at this pH in the ten liver or spleen homogenates belonging to the two control groups.

and spleen homogenates show a distinctly deviating picture: the two peaks are replaced by a single much less pronounced peak with maximal activity at pH 4·6. This change in the shape of the curve is seen for some of the mice in both the liver and the spleen homogenates. The shapes of the pH–activity curves for liver and spleen homogenates agree to some extent. Dependence of the shape of the pH–activity curves of β-glucuronidase in liver and spleen homogenates on the strain of mouse is also reported by Morrow *et al.*[9]

Injection with physiological saline produced no effect on the activity of the enzyme in the Swiss mice. After injection of the Swiss mice with solution of macromolecular substances, the activity of the enzyme in both liver and spleen homogenates increased over the whole pH range under investigation. It is noticeable, however, that the increase in activity after administration of solutions of macromolecular substances is smaller than that in the O_{20} mice. The increase in activity is relatively highest in liver homogenates of Swiss mice, as in the liver homogenates of O_{20} mice, in the pH range between 3·2 and 3·5. After administration of dextran, this increase is significant ($P \leqslant 0{\cdot}05$) between pH 3·2 and 5·1, and after PVP (50,000) it is significant ($P \leqslant 0{\cdot}05$) from pH 3·2 to 4·6. In the spleen homogenates the increase in activity for both substances is significant over the entire pH range studied ($P \leqslant 0{\cdot}05$). The relative increases of the activities in liver and spleen homogenates of the Swiss mice at the various pH values of the incubation media are given in Tables 3 and 4.

TABLE 3. β-GLUCURONIDASE, LIVER, SWISS MICE

(Activity relative to controls of the enzyme in liver homogenates of Swiss mice at various pH values of the incubation medium.)

Group	pH									
	3·2	3·5	3·9	4·3	4·6	4·8	5·1	5·4	6·0	7·0
I and II controls	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)
III dextran, m.w. = 200,000	1·7	1·4	1·3	1·3	1·3	1·3	1·3	1·2	1·2	1·2
V PVP, m.w. = 50,000	1·5	1·4	1·3	1·3	1·3	1·2	1·2	1·2	1·2	1·2
mean	1·6	1·4	1·3	1·3	1·3	1·3	1·3	1·2	1·2	1·2

TABLE 4. β-GLUCURONIDASE, SPLEEN, SWISS MICE

(Activity relative to controls of the enzyme in spleen homogenates of Swiss mice at various pH values of the incubation medium.)

Group	pH									
	3·2	3·5	3·9	4·3	4·6	4·8	5·1	5·4	6·0	7·0
I and II controls	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)
III dextran, m.w. = 200,000	1·6	1·6	1·3	1·3	1·4	1·3	1·3	1·4	1·3	1·5
V PVP, m.w. = 50,000	1·4	1·4	1·3	1·3	1·3	1·3	1·3	1·3	1·3	1·3
mean	1·5	1·5	1·3	1·3	1·4	1·3	1·3	1·4	1·3	1·4

Evidence of different peaks in the pH–activity curves of β-glucuronidase has also been obtained by other authors, mostly with partially purified enzyme preparations. Mills[10, 11] prepared two protein fractions from ox-spleen which exhibited optimal β-glucuronidase activity at pH 4·5 and 5·2, respectively. These findings were confirmed

by Kerr *et al.*[12] for purified liver and kidney preparations of mice. In later publications[6, 13] Mills *et al.* using ox-spleen and liver preparations separated three β-glucuronidase fractions having pH optima at 3·4, 4·5 and 5·2, respectively. These three pH optima thus agree well with our observations. A few of the experiments carried out by Mills indicated, however, that the fractions contained still another enzyme with an optimal activity at pH 6·3. The different fractions are regarded by Mills as constituting separate enzymes on the basis of : (a) the varying effects of altering substrate concentration on the velocity of hydrolysis; (b) differences in the energies of activation; and (c) the effects of various inhibitors. However, while Mills *et al.* consider that each of their enzyme fractions contains a distinct enzyme protein, the existence of several β-glucuronidases has been denied by a number of other authors[14–16] who believe that the fractions which have been separated are complexes of the same enzymic protein with different tissue components. Moreover, several authors report[5, 17] only one pH optimum for β-glucuronidase.

The activity of β-glucuronidase in the incubation medium can be raised or lowered by low concentrations of many different substances present in the homogenate. Carbohydrate derivatives in particular can reduce the activity of the enzyme.[7, 18, 19] Heparin, for instance, was found by Becker and Friedenwald[20] to inhibit the activity of β-glucuronidase by about 50 per cent at a concentration of 0·4 mg/100 ml. The inhibition was a function of pH.[6] Synthetic macromolecular substances have also been found to inhibit the enzyme, particularly at low protein concentrations,[21] while DNA, albumin, chitosan and certain diamines activate the enzyme.[22] Using clear homogenates obtained by centrifugation, we were able to demonstrate that dextran and polymers of vinylpyrrolidone can also inhibit the activity of the enzyme. This inhibition is rather complex and depends upon the concentration of the substrate and the concentration of the enzymes in the incubation medium. The inhibition is also dependent upon the type of substrate, the type of buffer solution and the pH. This made it desirable to investigate what quantities of the macromolecular substances were stored in the liver and spleen with the injection scheme applied in these experiments, in order to make it possible to determine what concentration of these substances is present in the incubation medium of the groups of mice which were injected with solutions of the macromolecular substances. These investigations[23] showed that the quantities of the macromolecular substances which were stored in the liver and spleen were too small to be capable of inhibiting the activity of the enzyme in the incubation medium. Triton-X-100 in the concentration used was found to increase the activity of the enzyme somewhat. For the pH range between 3·2 and 7·0, this activation amounted to less than 5 per cent. No differences in the amount of activation were found between the liver and the spleen homogenates and between the various groups of mice. The variations observed in the pH-activity curves of β-glucuronidase after administration of solutions of mocromolecular substances were thus caused exclusively by storage.

For the control groups, the quotients

$$\left[Q_{\text{liver}} = \left(\frac{\text{liver weight}}{\text{mouse weight}} \times 1000\right)^1\right]$$

for the O_{20} mice and Swiss mice were practically identical. In view of the fact that for control groups of both strains of mice the activity of β-glucuronidase per 100 g wet

liver tissue were also practically the same, the total enzyme activities in the liver, expressed per g mouse, showed little variation (Figs. 1 and 5).

In contrast to the livers, the quotients

$$\left[Q_{\text{spleen}} = \left(\frac{\text{spleen weight}}{\text{mouse weight}} \times 1000\right)^{1}\right]$$

for the control groups of both strains of mice differ appreciably. These quotients are about twice as large for the Swiss mice. Although the activity of β-glucuronidase per 100 g wet spleen tissue from the control animals of the O_{20} mice is higher than that of the Swiss mice, for the spleen the total enzyme activity expressed per mouse is higher in the Swiss mice than in the O_{20} mice (Figs. 2 and 6).

The storage of the macromolecular substances has been related to small cytoplasmic granules,[24–28] called dense bodies, peribiliary bodies, etc.[29] These type of granules, as far as they have been studied cyto- and bio-chemically, have been found to be rich in acid phosphatase and β-glucuronidase[30, 31] and are, in our opinion, identical with the lysosomes. β-Glucuronidase, however, is present not only in the lysosomes but also in the microsomes. De Duve found that in rat livers the pH–activity curves of β-glucuronidase present in the mitochondria–lysosome fraction have a different shape than those of the microsome fraction.[32] β-Glucuronidase deriving from the microsomes shows a maximal activity between pH 5·0 and 5·5. From pH 3·0 to 4·0 the activity is very low with respect to the β-glucuronidase deriving from the mitochondria–lysosome fraction. According to our opinion the macromolecular substances are stored in the lysosomes, and our investigations were intended to determine whether particularly the enzymes present in this organelles are increased in amount by the storage.

If this is the case, then on the basis of de Duve's observations concerning the differences in the shapes of the pH–activity curves of the various β-glucuronidase fractions it must be expected that the maximal relative increase in the activity of the enzyme complex after storage of the macromolecular substances must lie in the more acid range. This is indeed the case for the liver homogenates of both strains of mice.

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