PURIFICATION AND PROPERTIES OF A NEW β-D-GLUCURONIDASE

Thesis for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY

Julio Victor Peben

1963

This is to certify that the

thesis entitled

PURIFICATION AND PROPERTIES OF A
NEW β-D-GLUCURONIDASE

presented by

Julio Victor Pabon

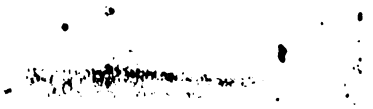
has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Chemistry

John C. Speck, Jr.
Major professor

Date August 19, 1963

O-169



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ABSTRACT

PURIFICATION AND PROPERTIES OF A NEW β-D-GLUCURONIDASE

By Julio V. Pabón

A new β-D-glucuronidase of high activity has been prepared from the digestive tract of the aquatic snail, Ampullaria cupina. The preparation of the enzyme involved (1) extraction with 20% saturated ammonium sulfate, (2) heat denaturation, (3) precipitation with ammonium sulfate, (4) fractional precipitation with ammonium sulfate, (5) fractional elution from DEAE-cellulose, and (6) ammonium sulfate precipitation. This procedure gave a 500-fold purification of the material in the first extract. The final fraction was colorless and amorphous. It was free of arylsulfatase activity and probably was free of cellulase activity also. Repeated precipitations of the preparation with controlled ammonium sulfate concentrations yielded an electrophoretically homogeneous fraction of very high activity. Examination of a purified preparation in the analytical ultracentrifuge allowed the approximation of the molecular weight of the enzyme. The sedimentation data obtained at top speed of the rotor indicated that the preparation obtained by the purification procedure (steps 1-6) was about 63% pure.

The catalytic properties of the enzyme have been investigated. The enzyme hydrolysed readily phenolphthalein, pregnanediol, 1-menthyl, and p-nitrophenyl glucuronides. The enzyme exhibited a sigmoid pH-activity curve with inflection point about pH 4.7. The Michaelis constant, determined for phenolphthalein glucuronide, was found to vary

with the pH of the assay mixtures. The constant, however, was independent of pH when determined for p-nitrophenyl glucuronide. The enzyme was inhibited strongly by mercuric ion, and moderately by silver and cupric ions. Sulfhydryl group reagents caused no inhibition. The effect of other parameters, such as temperature, nature of buffering ions, stability of the enzyme and nature of the aglycons, on the enzyme activity has been determined also.

PURIFICATION AND PROPERTIES OF A NEW β-D-GLUCURONIDASE

By

Julio Victor Pabón

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Chemistry

1963

ACKNOWLEDGMENT

The author wishes to express his sincere thanks to Dr. John C. Speck, Jr., whose inspiration, great interest, guidance and co-operation made possible the progress of the present investigation.

Grateful acknowledgment is also due to Mr. Harold Swaisgood for running the ultracentrifugation experiments and making the necessary calculations.

He is also indebted to Mr. Richard Dardas for his kind help in checking the homogeneity of one of the purified enzyme preparations.

Acknowledgment is also due to the Department of Chemistry, the Department of Biochemistry and the National Institutes of Health for funds provided in support of this work.

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PURIFICATION AND PROPERTIES OF A NEW β-D-GLUCURONIDASE

I. INTRODUCTION

All mammalian tissues and fluids contain group-specific enzymes known as β-glucuronidases[1]. β-Glucuronidases have been obtained also from diverse sources other than mammalian tissues. They catalyze the hydrolysis of the β-D-glucopyranosiduronic acids of all types to the aglycons and D-glucuronic acid. The presence of D-glucuronic acid in hydrolyzates have been established by several ways (3), and it has been shown in experiments carried out in H_2O^{18} that hydrolytic cleavage occurs at the glycosyl-O bond (5). Fishman and Green (6) have shown that these enzymes exhibit transferase activity also; thus, liver, bacterial and molluscan β-glucuronidases catalyze transfer of the β-D-glucuronosyl group from aryl and cycloalkyl β-D-glucuronides to aliphatic alcohols and glycols but not to phenols or alicyclic alcohols.

The function of these enzymes in the animal body is still a subject of speculation. Its wide distribution in the mammalian tissues and fluids, coupled with changes in activity that have been observed in various physiological and pathological processes, suggests that they may play important metabolic and physiological roles. The contention, held for some time, that β-glucuronidases were responsible for the synthesis of glucuronides in vivo has been abandoned (6). The now established pathway for glucuronide synthesis employs uridine 5-(D-glucosyluronic acid dihydrogenpyrophosphate) and the enzyme glucuronyltransferase (7,8). The presence of β-glucuronidase activity in crude

[1]For recent reviews of the β-glcuronidase literature the reader should consult references 1-4.

testicular hyaluronidase preparations (9), and the action of this glucuronidase (or these glucuronidases) on oligosaccharides released by hyaluronidase from hyaluronic acid and chondroitin sulfate (10), suggest that they may play a role in the mucopolysaccharide catabolism. This role is also suggested by the changes in glucuronidase activity which have been observed in certain organs in response to free hormones (in vivo), in common with other mammalian glycosidases that also act on degradation products of hyaluronic acid (11).

Historical Background

The first report of the decomposition of β-glucuronides by plant and bacterial preparations appeared in 1904 (12). It was reported that raw emulsin from almonds and extracts from the kefir grains contained a splitting enzyme for conjugated glucuronides. This finding was confirmed in 1906 (13) and in 1907 (14). In 1908 appeared the first report of decomposition of conjugated glucuronides by a mammalian preparation (15); menthol glucuronide was hydrolyzed by a dog liver extract. Hämäläinen (16) reported in 1910 that yeast extracts did not hydrolyze borneol- and camphorglucuronic acids. Sera (17) reported in 1914 that orcinol and phloroglucinol glucuronides were decomposed by mammalian extracts; these glucuronides, as well as vanillin glucuronide, were not split by emulsin (18, 19). Later, Ishidate (20) showed that menthol and p-hydroxycamphorglucuronic acids could be hydrolyzed by means of emulsin if proper conditions of pH and temperature were maintained. In 1933, Helferich and Sparmberg (21) found that the hydrolysis of l-menthol-β-D-glucuronide by almond emulsin in different stages of purification did not parallel that of l-menthol-β-D-glucoside.

It was assumed that a specific enzyme was present in emulsin for the cleavage of the glucuronides.

Masamune (22), in 1934, commenced the systematic study of β-glucuronidases with the characterization of the enzyme from beef kidney. In that year, Oshima (23) reported the distribution of β-glucuronidase in the tissues of the dog and the ox, and later in 1936, the purification of the enzyme from ox-spleen (24). The purification procedure consisted of autolysis followed by adsorption on Kaolin at acid pH and elution at alkaline pH. At the same time, Hofmann (25) studied the hydrolysis of l-menthol and β-naphthol β-D-glucuronide by mammalian extracts while investigating the specificity of mammalian glycosidases; earlier inconsistencies were explained. In 1939, Fishman (26, 27) started studying the mammalian β-glucuronidase to determine whether the enzyme was concerned with the synthesis of estriol glucuronide in the female organism. He reported a 137-fold purification of the enzyme from ox-spleen and investigated the action of the purified enzyme on borneol, menthol, and estriol β-glucuronides. The ox-spleen enzyme was again purified by Graham (78) in 1946. A 315-fold purification was obtained by a procedure which involved mincing in water, precipitation of proteins with acetone, and ammonium sulfate fractionation at three different pH values.

The study of β-glucuronidases was greatly facilitated when a colorimetric method of assay which utilizes phenolphthalein mono-β-D-glucuronide as substrate was introduced in 1946 (28). Mills (29), in 1948, showed the presence in spleen extracts of two protein fractions having β-glucuronidase activity. The two fractions were separated and purified by ammonium sulfate fractionation. The two fractions exhibited

different pH optima at pH 4.5 and pH 5.0. This was confirmed by Kerr et al. (30) in the liver and kidney of the mouse. Sarkar and Sumner (31) employed dioxane fractionation, calcium phosphate adsorption, and ammonium sulfate fractionation to purify the enzyme from ox-liver. A 6000-fold purification was reported but it appears that the activity of the purified fraction was 31,000 Fishman Units per mg. of protein (37).

Jarrige and Henry (32) found the digestive juice of the land snail, Helix pomatia, a very rich source of the enzyme and studied some of the properties of the enzyme. Locusts (33) and marine molluscs (34) were shown to contain large amounts of the enzyme also.

Mills et al. (35), continuing with the investigation of the β-glucuronidase from ox-spleen, presented evidence in 1953 for the occurrence in this tissue of three glucuronidase fractions with pH optima at pH 3.4, pH 4.5, and pH 5.2. This work has not been confirmed.

In that same year, Smith and Mills (36) purified the enzyme from ox-liver by applying a procedure involving metallo-protein reactions, celite adsorption, and elution from celite with ammonium sulfate solutions of different concentrations. The purified preparation has a specific activity of 32,000. Then, Fishman et al. (37), employing alkaline ammonium sulfate fractionation followed by anion exchange and methanol fractionation, obtained a preparation from calf liver with specific activity of 107,000. This preparation was regarded as 85 per cent pure on the basis of physical tests of homogeneity.

Recently, a new approach was adopted for the purification of the mammalian enzyme (38). Instead of a tissue readily available, but with low enzyme activity, the tissue with the highest activity (female rat preputial gland), was chosen as the starting material. The gland,

which is very small, has an initial specific activity of about 18,000. After a simple fractionation procedure employing ammonium sulfate and ethanol, a preparation was obtained with a specific activity of 455,000. The preparation was colorless and was stable for at least 18 months when buffered at pH 5.0.

Alfsen and Jayle (39) have reported the only crystalline β-glucuronidase preparation. It was obtained from the land snail, Helix pomatia, and exhibited a specific activity of 120,000. The purification procedure employed ammonium sulfate fractionation followed by ethanol fractionation. The preparation was found homogeneous by electrophoresis and analytical ultracentrifugation. Later, Wakabayashi and Fishman (40) described an improvement in the method of Alfsen and Jayle which gave a preparation free of sulfatase activity, although not of greater β-glucuronidase activity. The method (heat denaturation) of freeing β-glucuronidase from arylsulfatase (41) yielded a preparation from the limpet, Patella vulgata, also almost free from arylsulfatase (42).

Still, a pure β-glucuronidase preparation needs to be obtained in sufficient amounts to permit a complete study of the enzyme properties. For this it is necessary to find a source readily available and from which the enzyme could be isolated by a simple procedure. Although these enzymes are widely distributed in mammals and molluscs very few highly purified preparations have been obtained. The best preparation of these enzymes is the one from the richest source, the rat preputial gland, but this source presents the inconvenience of being very small and requiring a delicate technique for its separation from other tissues. The viscaral hump of the limpet or the digestive tract of the snail appears to be the most promising sources for these enzymes since

these are the richest alternatives to the rat preputial gland (3). Previous work in this laboratory (43) suggested that the digestive tract of the aquatic snail, Ampullaria cupina, might be a good source for one of these enzymes. The purpose of the present work was to look further into the isolation of the enzyme from this source, improve the purification procedure, and study some of the properties of the purified enzyme.

Occurrence

The distribution of β-D-glucuronidases is general in mammalian tissues and body fluids. Highest in activity are the liver, kidney, spleen, epididimis, and cancer tissues. The distribution of these enzymes is probably general also in other vertebrates, as well as in insects and molluscs (a comprehensive list of known sources of these enzymes is found in reference 3). The digestive juices or digestive tracts of molluscs are specially rich sources of these enzymes. They occur sporadically in plants and are randomly distributed in bacterias.

The distribution of these enzymes within the cell has been studied in homogenates of mouse and rat tissues (3). The enzyme in isotonic homogenates of mammalian tissues (44) was sedimented with the mitochondrial and microsomal fractions; little was present in the nuclear fraction or free in the cytoplasm. When mammalian tissues were homogenized in water (45), more than half of the enzyme escaped into solution and the residue within the granules was completely accessible to substrate.

Assay of Enzyme Activity

There is a variety of sensitive and specific methods, employing different substrates, for the measurement of β-glucuronidase activity (for description of most convenient methods see reference 3). Most of

the methods of assay depend upon colorimetric determination of the amount of aglycon liberated from a specified concentration of substrate at a fixed pH value. The unit of enzyme activity has been almost universally expressed as that which liberates 1 μg of aglycon or D-glucuronic acid in 1 hour at 38°. When the substrate used is phenolphthalein mono-β-D-glucuronide, the most commonly used, this unit is known as the Fishman Unit. When it is more convenient to measure the liberated glucuronic acid this can be effected by the method of Fishman and Green (46) which is based on the Tollens naphthoresorcinol color reaction for uronic acids. Recently, p-nitropheny1 β-D-glucuronide was synthesized (47) and it has been suggested as a better substrate than phenolphthalein mono-β-D-glucuronide (48).

Variation of the Activity with pH

A list of the pH optima for hydrolysis of β-glucuronides by mammalian and non-mammalian β-glucuronidase preparations has been compiled (3). Mean values for the optimum pH are 4.5 and 5.2. Mammalian preparations might exhibit either or both depending on the source and purity of the enzyme, and on the conditions of the assay.

Non-mammalian preparations of these enzymes display only single pH optima. The different enzyme preparations appear to fall in two groups: (a) the bacterial preparations with optima near neutrality and (b) the remaining preparations with optima below pH 5.0.

Mammalian and non-mammalian β-glucuronidase preparations have shown little change in the pH optima for glucuronides of different aglycons in agreement with the finding (49) that the nature of the aglycon has little effect on the ionization of a glucuronide. However,

the pH optima for the hydrolysis of phenyl β-D-galacturonide by the rat preputial gland preparation was 3.9 while that for the phenyl glucuronide was 4.5 (50).

pH-Stability and the Effect of Temperature

The mouse liver β-glucuronidase was found pH-stable between pH 5.0 and pH 7.0 at 0°C (51). The range of stability of the limpet β-glucuronidase was greater by one pH unit on both the alkaline and acid sides of neutrality (51). A period of contact with the acid or base of one minute or one hour made no difference with this limpet preparation. However, raising the temperature to 37° narrowed the stability range. Another molluscan preparation has shown the same stability that the limpet preparation did (52). Rumen β-glucuronidase was stable only at the pH region of maximum activity (53).

The ox-liver β-glucuronidase was stable to 30 minutes of heating at 50° (31). It was not stable above this temperature. The energy of activation for the β-glucuronidase of human urine was -13,600 cal./mole (54), and for the enzyme from H. pomatia this value was -14,200 (55). Mammalian preparations have been reported to double their activity for every 10° rise in temperature (24, 31, 35). This is probably true for molluscan preparations also (56).

Interfering Substances

A variety of natural and synthetic substances have been shown to interfere with the β-glucuronidase activity (3, 4). Heparin, chondroitin sulfate, and hyaluronic acid are weak, non-competitive inhibitors. Ionic resins inhibit the enzyme non-competitively also, and the inhibition

by detergents (alkyl sulfates) rapidly becomes irreversible, with complete inactivation of the enzyme.

Highly purified mammalian β-glucuronidase preparations when highly diluted appear to lose activity, but they are activated by albumin, deoxyribonucleic acid, chitosan, heat inactivated glucuronidase, starch, suramin and certain diamines (36, 57). Albumin showed no effect on the position of the pH optima, but deoxyribonucleic acid shifted the pH optima to the alkaline side of the pH-activity curve (38, 36). Phthalic acid produced the same type of effect as deoxyribonucleic acid (36).

An unidentified, non-dialyzable and thermostable agent which has the capacity of inhibiting the mammalian enzyme has been reported present in the blood plasma (58) and in aqueous rat liver suspensions (59). The effect of this inhibitor was not pH-dependent.

Recently, a new unidentified agent which is also non-dialyzable and thermostable has been extracted from water-insoluble cell debris of a rat liver homogenate. It has the ability to activate calf liver β-glucuronidase (60).

The effects of cations on the β-glucuronidase activity have been studied for mammalian and non-mammalian preparations (3, 61). Only cupric, silver and mercuric ions have been found strong inhibitors. The action of cupric ion was weak unless potentiated by a reducing agent such as L-ascorbic acid or sodium bisulfite (61, 62). These substances had no effect on the inhibition by silver and mercuric ions. It was observed that low cation concentrations caused slight activation of crude mouse and rat liver preparations (63). The inhibitory action of silver, cupric and mercuric ions on the rat preputial gland preparation appeared to be primarily competitive (61).

Inhibition by p-chloromercuribenzoic acid has been reported for a bacterial preparation of these enzymes (64) and for the rat preputial gland preparation (61). The inhibition of the bacterial enzyme was reversed by cysteine; that of the mammalian enzyme was competitive.

Specificity and Inhibition

The mammalian β-glucuronidase preparations investigated appear to catalyze the hydrolysis of all natural and synthetic β-D-glucopyranosiduronic acids whether aliphatic or aromatic (3, 48, 54). They also catalyze the hydrolysis of 1-O-acyl-β-D-glucuronic acids (65, 66), and of β-D-glucopyranosiduronic acid-1-phosphate (67). The comparative ability of mammalian, molluscan and bacterial enzyme preparations to hydrolyze steroid β-glucuronides has been studied recently (40, 68, 69). There was no evidence for a clear superiority of any one of the enzyme preparations for all the steroid glucuronides studied. The mammalian enzyme has no action on α-D-glucopyranosiduronic acids or on β-D-glucopyranosides. Probably, non-mammalian preparations exhibit a similar behaviour (3, 55).

Whether these enzyme preparations can catalyze hydrolysis of β-D-glucuronides with a furanose ring is still unsettled, since substances having this structure have not been prepared. It is of interest, however, that Nakao *et al.* (70) reported that p-aminobenzoyl-β-D-glucuronide has a furanose structure. This glucuronide is hydrolyzed by β-glucuronidase (66). The glycosides of D-glucofuranuronolactone are not attacked.

β-glucuronidases from all sources often display marked inhibition in the presence of excess substrate.

Hydrolysis of β-glucuronides by mammalian or non-mammalian enzyme preparations is powerfully and competitively inhibited by D-glucaro-1,4-lactone (71). The inhibition is pH-dependent (54, 72); it decreases with increasing pH. D-glucaro-1,4-lactone is a highly specific inhibitor for β-glucuronidases (72), and it was potent inhibitor of the transferase activity of these preparations also (6). Inhibition by solutions of D-glucaric acid was shown to be due to D-glucaro-1,4-lactone present in the solutions (71).

D-Glucarolactone requires a free carboxylic acid group at the 6-position to be an effective inhibitor. D-glucaro-6,3-lactone and D-glucuronolactone do not appear to inhibit the glucuronidase preparations (73). The affinity of D-glucaro-1,4-lactone was shown to be 1000-times as great as that of its 6-methyl and 6-ethyl esters (54).

Solutions of galactaric acid have been shown to inhibit β-glucuronidase preparations of mammalian and molluscan origin markedly (35, 71, 72, 73). Solutions of D-glucuronic acid also inhibit the enzyme, although to a lesser extent (71).

The inhibition of β-glucuronidase preparations by solutions of galactaric acid was considered an anomalous behaviour until it was found that mammalian and limpet enzyme preparations hydrolyze β-D-galactopyranosiduronic acids (38, 50). The identity of β-glucuronidase and β-galacturonidase was suggested by the action of specific inhibitors and by the high activity of preparations from rat preputial gland and from P. vulgata toward both types of substrate. The two activities in P. vulgata preparations displayed identical pH-stability. The preparations from the limpet, unlike the mammalian preparations, hydrolyzed α-glucuronides also (50, 74). The enzyme responsible to

the hydrolysis of α-glucuronides was distinguished from β-glucuronidase since it displayed different pH and heat stability and was not inhibited by glucaro-1,4-lactone or galactarolactone.

β-D-glucuronidase activity has been found present in crude, testicular hyaluronidase preparations (9). It is believed that hyaluronic acid is composed predominantly of alternate, β-linked, D-glucuronic acid and N-acetyl-D-glucosamines residues (75); chondroitin sulfate is a similar type of polymer in which the amino sugar is N-acetyl-D-galactosamine. Testicular hyaluronidase degrades hyaluronic acid and chondroitin sulfate to oligosaccharides from which, as has been shown (9, 10), β-glucuronidases and β-N-acetylhexosaminidase split off alternately D-glucuronic acid and hexosamine from their non-reducing ends. Considering this behaviour, the enzyme may be regarded an exo-β-D-glucuronidase.

Recently, it has been reported that the hyaluronidase from the medicinal leech is an endo-β-D-glucuronidase (75, 76). This enzyme hydrolyzes hyaluronic acid to aligosaccharides which have the uronic acid moiety on the free reducing end. More recently, this enzyme has been purified by Yuki and Fishman (77). The specificity of the enzyme for hyaluronic acid appears to be unique and exclusive for the endo-β-glucuronide linkage. Simple glucuronides were not hydrolyzed. Crude extracts contained the exo-β-D-glucuronidase but not the purified fraction. Other properties also indicated that this enzyme is not identical to the exoglucuronidases.

II. EXPERIMENTAL

1. Apparatus

Spectrophotometer. — Absorbance measurements in the visible range were carried out in the Beckman Model B spectrophotometer. The cell compartment was replaced with a test tube compartment.

Centrifuges. — The International Centrifuge Model HR-1 was used for preparative purposes. The Spinco Model E Analytical Ultracentrifuge was used to study the sedimentation properties of a purified enzyme preparation.

Columns for Fractional Elution of Proteins. — Columns fitted with fritted glass discs (coarse) or glass-wool plugs and packed with the desired resin were employed in the purification of β-glucuronidase.

Zone Electrophoresis.— Experiments on paper (Whatman #1) were carried out in the Paper Electrophoresis Apparatus Type L. K. B. 3276 (Ivan Sorvall, Inc.). The experiments on oxoid cellulose-acetate strips (Consolidated Lab., Inc.) were carried out in the Shandon Electrophoretic Cell (Shandon Scientific Co., London).

Standardized Test Tubes. — Soft-glass test tubes were standardized by comparing the absorbance reading of an alkaline phenolphthalein solution when transferred from one tube to another. The roundness of the tubes was checked by rotating the tubes in the instrument.

2. Materials

Snails. — Aquatic snails, Ampullaria cupina, were obtained from the Streamland Aquarium, Florida.

Protein. — Bovine serum albumin, 3x crystallized, was purchased from Pentex, Inc.

Resins. — Sephadex G-75 (Lot To 8492 M) and diethylaminoethyl-sephadex-A50 (Lot To 7874 M) were purchased from Pharmacia (Sweden). Diethylaminoethyl-cellulose (Lot 107418, 0.78 meq. per g; Lot 500391, 0.62 meq. per g) and diethylaminoethyl-Solk-Floc (Lot 104169, 0.50 meq. per g) were obtained from California Corporation for Biochemical Research.

Chemicals. — p-Chloromercuribenzoate (sodium salt, Lot 102735) was obtained from California Corporation for Biochemical Research. N-Ethyl maleimide (Lot c2282) was obtained from Mann Research Lab.

Reagents. — (a) Folin-Ciocalteu phenol reagent was obtained from the Hartman-Leddon Co.

(b) Folin-Ciocalteu alkaline reagent: 73 g of anhydrous sodium carbonate in 1927 ml. of water; 0.6 g of cupric sulfate pentahydrate in 19.4 ml. of water; and 1.05 g of NaKtartrate tetrahydrate in 18.9 ml. of water. The sodium carbonate solution is made first and filtered. The cupric sulfate and tartrate solutions are mixed and added to the carbonate solution.

(c) 0.4% aqueous naphthoresorcinol solution: The naphthoresorcinol is pulverized and suspended in water. The suspension is shaken

for ten minutes in an amber colored mixing-cylinder. The filtered solution is kept away from light. A fresh solution is prepared every day.

Buffers. — (a) 0.1M phosphate buffer, pH 7.0 -- 28.8 g of sodium monohydrogenphosphate (anhydrous) and 8.16 g of sodium dihydrogen phosphate monohydrate were dissolved in 1500 ml. of water, the pH was adjusted if necessary and then it was diluted to 2000 ml.

(b) 0.1M acetate buffer, pH 4.5 -- 8.2 g of sodium acetate and 7.73 ml. of glacial acetic acid were put in 700 ml. of water, the pH was adjusted if necessary and then it was diluted to 1000 ml.

(c) 0.2M glycine-NaOH buffer, pH 10.4 -- 15 g of glycine and 11.7 g of sodium chloride were dissolved in 700 ml. of water. The pH was adjusted to 10.4 with 10% NaOH solution and the mixture was diluted to 1000 ml.

(d) Carbonate buffer, pH 10.1 -- 8.4 grams of anhydrous sodium bicarbonate and 36 g of anhydrous sodium carbonate are dissolved in a liter of water.

(e) 0.1M phthalate buffers ofvarious pH values (37°C) -- 50 ml. aliquots of 0.2M KHphthalate solution were adjusted to the desired pH values with 1M HCl or 1M NaOH solutions at 37° and diluted to 100 ml.

(f) 0.1M acetate and citrate-phosphate buffers of various pH values -- The buffers were prepared as described in Methods in Enzymology, Vol. I, pages 138-46. Each buffer solution was checked in the pH meter and if necessary, adjusted to the desired pH with dilute acid or base solutions.

Substrates. — Phenolphthalein mono-β-D-glucuronide (Lot 51 B-690), borneol-β-D-glucuronide (Lot 111 B-801), l-menthol-β-D-glucuronide (Lot 36-40), and pregnanediol-β-D-glucuronide were purchased from Sigma Chemical Co.

3. Preparation of p-Nitrophenyl-β-D-Glucuronide (47)

The method of Kato et al. was slightly modified for this preparation: 8.0 g of methyl(tri-O-acetyl-α-D-glucopyranosyl bromide)-uronate (79) and 14.0 g of p-nitrophenol were dissolved in 100 ml. of acetonitrile. 4.0 g of silver oxide were added and the suspension was shaken overnight. The reaction mixture was filtered. The filtrate was collected in 100 ml. of chloroform. The silver salts were extracted with 50 ml. of chloroform and the extract was combined with the chloroform solution containing the bulk of the product. The chloroform solution was extracted three times with water, then three times with 2M KOH solution, and again with water. The mixture was dried over drierite and evaporated under diminished pressure. The residue was crystallized twice from isopropyl alcohol. Yield of methyl(p-nitrophenyl-tri-O-acetyl-β-D-glucopyranosid)-uronate: 50%. m.p. 150-52°

Methyl(o-nitrophenyl-tri-O-acetyl-β-D-glucopyranosid)-uronate was obtained by this procedure from 10 g of methyl (tri-O-acetyl-α-D-glucopyranosyl bromide)-uronate. The product was crystallized from acetone. The yield was 70%. m.p. 175-76°.

7.32 g of methyl(p-nitrophenyl-tri-O-acetyl-β-D-glucopyranosid)-uronate were dissolved in 50 ml. of 0.1M sodium methoxide solution by stirring with a swirling motion. The solution was allowed to stand at room temperature for 20 hours. The methanol was evaporated under

diminished pressure. The residue was taken up in 40 ml. of 0.43N barium hydroxide solution and allowed to stand at room temperature for one hour. Then, cation exchange resin (IR-120) was added and the suspension was shaken until a clear solution of pH about 2.5 was obtained. The suspension was filtered and the filtrate was evaporated under diminished pressure. The solid residue was dissolved in ethyl acetate which previously had been shaken with water in a separatory funnel. Ether was added to the solution until it became permanently cloudy, the flask was stoppered, and allowed to stand at room temperature. After one to three days the compound crystallized. The product (p-nitrophenyl-β-D-glucuronide) was collected on a filter and dried in a vaccum dessicator over phosphorus pentoxide. m.p. 95°. Specific rotation at 23°C, D line of Na, was equal to -112° (water, 0.2836). This product corresponds to the monohydrate compound.

Repeated attempts to obtain o-nitrophenyl-β-D-glucuronide by this procedure were unsuccessful.

4. Determination of Protein

Folin-Ciocalreu Phenol Reaction (80). — An aliquot of the protein solution is placed in a soft-glass test tube and the volume is brought to 1.0 ml. with water. 5.0 ml. of the alkaline reagent are added to the tube and this is incubated at 37° for 20 minutes. The phenol reagent is diluted (1:2) and 0.5 ml. are added to the tube; the contents are mixed immediately. The absorbance at 660 mμ is read after 30 minutes of standing at room temperature. A bovine serum albumin solution (1 mg. per ml.) was used to prepare standard curves (O.D. at 660 mμ versus μg of protein).

5. Assay of Enzyme Activity

(a) Phenolphthalein mono-β-D-glucuronide. — The β-D-glucuronidase activity was assayed with this substrate by the method of Fishman et al. (28) slightly modified. Each determination was run in duplicate with a single control. Controls on the spontaneous hydrolysis of the substrate always showed that the amount of hydrolysis occurring under the conditions of the tests was undetectable. The assay mixtures consisting of 0.5 ml. of 0.1M acetate buffer, pH 4.5, 0.5 ml. of 0.0015M substrate solution, and 0.5 ml. of enzyme dilution were incubated at 37° for exact periods of time (usually 30 minutes). Bovine serum albumin (100 μg per ml.) was added to the dilutions of highly purified enzyme preparations; this was not necessary with crude preparations. The reaction was stopped by the addition of 5.0 ml. of 0.2M glycine buffer, pH 10.4, and the optical density was read at 540 mμ with the Beckman Model B spectrophotometer. For the determination of the initial velocity of reaction by this procedure the incubations were done for only several minutes (counted after the first minute in the water bath), and a complete assay mixture, incubated for one minute, was used as the blank for the reading of the optical density.

In the present work the β-D-glucuronidase activity is expressed in phenolphthalein units. One such unit was defined as the activity which liberates one microgram of phenolphthalein per hour in acetate buffer, pH 4.5, at a temperature of 37°, and a 0.0005M substrate concentration. This unit is not identical to the Fishman Unit since it is not defined for the pH of optimum enzyme activity. Although the enzyme is more active at pH values below 4.5 (Figure 5), the assay of the

activity at pH 4.5 was convenient because the enzyme has great stability at this pH (Table VII).

(b) p-Nitrophenyl-β-D-Glucuronide. — The assay procedure used for the phenolphthalein glucuronide was applied to this substrate, but the optical density was read at 400 mμ.

(c) l-Menthol-, d-Borneol-, and Pregnanediol-β-D-Glucuronides. — 0.5 ml. of the substrate solution, 1.0 ml. of 0.1M phthalate buffer of the desired pH, and 0.5 ml. of enzyme dilution were mixed. 0.5 ml. of boiled enzyme were added to the controls. The mixtures were incubated (at 37°) for exact periods of time at the end of which the tubes were immersed in boiling water for one minute. The extent of hydrolysis was determined employing the method of Fishman and Green (46) for determination of free and conjugated glucuronic acid. The method depends on carrying out the naphthoresorcinol reaction before and after the oxidation of the free glucuronic acid by hypoiodite to saccharic acid at pH 10.1. The difference in values obtained for glucuronic acid before and after the oxidation procedure gives a measure of the unconjugated glucuronic acid.

The assay mixtures were diluted with water so that the concentration of total glucuronic acid (free plus conjugated) was not more than 20 μg per ml. Aliquots of 5.0 ml. were put in 50 ml. erlenmeyer flasks containing 2.05 ml. of carbonate buffer, pH 10.1. 1.5 ml. of 0.1N iodine solution were added, shaken gently, and the flasks were stoppered and allowed to stand in the dark for 30 minutes. At the end of this, 0.15 ml. of 1.0M sodium bisulfite solution were added, the flasks agitated, and an addition made of 0.3 ml. of 6N sulfuric acid. Any

residual iodine coloration was removed by one additional drop of bisulfite solution. The flasks were shaken to remove the excess carbon dioxide from the solution. This mixture yielded the value for glucuronide glucuronic acid.

To obtain the figure for total glucuronic acid, another 5.0 ml. of solution was pipetted into a solution containing iodine, bisulfite, and sulfuric acid prepared in the amounts and sequence as before.

Four ml. aliquots (in duplicate) were then pipetted into pyrex test tubes (capacity about 50 ml.). To each were added 2.0 ml. of 0.4% naphthoresorcinol solution and 2.0 ml. of 18N sulfuric acid. The contents of the tubes were mixed well, and the tubes, unstoppered, were placed in a boiling water bath for one hour. The tubes, still in the rack, were immersed in cold water. After cooling, 10 ml. of 95% alcohol were added to each tube, the tubes were shaken to dissolve the pigment, and 8 ml. of toluene were added. Cork stoppers were inserted and the tubes were vigorously shaken 100 times to extract the violet pigment into the toluene phase. The aqueous layer was removed by suction with a tube drawn out to a capillary attached to an aspirator and collecting bottle. The toluene extracts were then transferred into standardized soft-glass test tubes. After allowing the extracts to stand in the dark (5 minutes) to permit them to clear, the optical density of each tube was measured at 565 mμ (0 optical density with a reagent blank). The reagent blank should not read below 85% transmittance (0 optical density with a toluene blank).

Calibration Curve: 5.0 ml. of solutions that contain 1.25, 2.5, 5.0, 10, and 16 μg of glucuronic acid per ml., respectively, are pipetted

into erlenmeyer flasks that already contain the previously stated amounts of buffer, iodine, bisulfite, and acid (final volume, 9.0 ml.). Four ml. aliquots of the mixtures (in duplicate) are then pipetted for the naphthoresorcinol reaction. The concentration of glucuronic acid in the 4.0 ml. aliquots is plotted against optical density to yield a straight line.

6. Purification of the Enzyme

Step 1: Extraction. — The shells of the snails (killed in batches of 150 to 200), Ampullaria cupina, were removed with scissors. The foot, the respiratory system and reproductive tract were cut and discarded. The remainder of the snails, consisting primarily of the hepato-pancreas, intestines and crop, was put in cold 20 per cent saturated ammonium sulfate solution (300 ml.). The cold suspension was homogenized in a waring-blender (1 minute). The homogenate was centrifuged in a refrigerated International Centrifuge Model HR-1 at 11,000 rpm (all preparative centrifugations were done in this machine). After 15 minutes of centrifugation, the sedimented material was extracted with additional ammonium sulfate solution. The two extracts combined made about 500 ml. of a brown mixture (fraction I). The insoluble residue was discarded without further treatment.

Step 2: Heat denaturation. — Wakabayashi and Fishman (41) obtained the β-D-glucuronidase from Helix pomatia free of sulfatase activity by heat denaturation. By the same procedure, our extract was heated to 70-74° and kept at that temperature for four minutes. Then, the suspension was cooled in an ice bath. When cold, then suspension was centrifuged at 11,000 rpm for 20 minutes. The sedimented precipitate

was extracted once with 20 per cent saturated ammonium sulfate solution and then discarded (fraction II). The combined supernatants made about 550 ml. (fraction III).

Step 3: Ammonium sulfate precipitation. — Fraction III was adjusted to 55 per cent salt saturation with solid ammonium sulfate. The suspension was stirred for 30 minutes and then centrifuged at 11,000 rpm for 20 minutes. The supernatant (fraction IV) was discarded. The precipitate (fraction V) was dissolved in 150 ml. of water and the mixture allowed to stand in the cold overnight. The material that sedimented was removed by centrifugation at 16,000 rpm for 30 minutes. The solid (fraction VI) was discarded. The supernatant was dialysed in 20 per cent saturated ammonium sulfate solution in the cold room (2 days, 3 changes of salt solution). If more material sedimented, it was removed by centrifugation and discarded.

Step 4: Fractional precipitation with ammonium sulfate. — The dialysed mixture (fraction VII) was adjusted to 38 per cent salt saturation with solid ammonium sulfate and stirred for 30 minutes. The precipitated protein (fraction VIII) was removed by centrifugation (16,000 rpm) and discarded. The supernatant (fraction IX) was further adjusted to 50 per cent saturation, stirred for 30 minutes and centrifuged at 16,000 rpm for 20 minutes. The supernatant (fraction X) was discarded. The precipitated protein (fraction XI) was diluted to 25 ml. and put to dialyse in 0.005M phosphate buffer, pH 7.0.

Step 5: Fractional elution from DEAE-cellulose. — The diethylaminoethyl-cellulose (22 g) was suspended in 0.005M phosphate buffer, pH 7.0, stirred several minutes and filtered by suction. The slow sedimenting particles of the resin were discarded by suspending the

resin in the buffer, allowing the fast sedimenting particles to settle and decanting the supernatant fluid with the slow sedimenting particles; this was repeated several times. The remaining slurry was put in a filtering flask and the entrained air removed in the water pump. The slurry was packed to a height of about 30 cm. (into a glass cylinder 2.5 x 37 cm.). The column was put in the cold room and 0.005M phosphate buffer was allowed to flow through it overnight from a 500 ml. capacity separatory funnel used as solvent reservoir. The crude enzyme preparation (usually between 250 and 400 mg. of protein) was placed in the column. Then, the column was washed with 500 ml. of 0.005M phosphate buffer, pH 7.0, at a rate of 1 ml. per minute and collecting fractions of 10 to 15 ml. When the washing elution was completed, the buffer was changed to 0.01M phosphate buffer, pH 7.0, and the column was eluted with 250 ml. of the buffer. The fractions containing the bulk of the activity were combined (usually made 25 to 40 ml.).

Step 6: Ammonium sulfate precipitation. — The DEAE-cellulose eluate was adjusted to 60 per cent salt saturation with ammonium sulfate, stirred for 30 minutes, and centrifuged at 17,000 rpm. The supernatant was discarded. The precipitate was dissolved in a few ml. of water and centrifuged at 17,000 rpm for 15 minutes. The insoluble matter was discarded. The supernatant was adjusted to 60 per cent salt saturation with ammonium sulfate and stored in the refrigerator.

7. Zone Electrophoresis

The homogeneity of the most purified enzyme fraction (specific activity 168,000) was tested by paper electrophoresis (81, 82). The Whatman #1 filter paper strips (2.5 x 47 cm.) were saturated with the desired buffer by passing them through the buffer; each strip was

blotted with another dry strip to remove excess buffer. Twenty to 30 µl. of the enzyme solution (1.16 mg per ml.) were applied on the paper either directly with a small pipette or the sample was first transferred from the pipette to the sample applicator (SA 3276). Either way of application produced a narrow band of the sample on the mid-point of the strip. The strips were placed in the apparatus (L.K.B. 3276) located in the cold room and a field of 270 volts (10 ma.) was applied for the desired period of time. Experiments were carried out at pH 3.0 (0.1M glycine-HCl) and pH 3.8 (0.1M acetate) for 4.5 hours; pH 4.5 (0.1M acetate) for 9.5 hours; pH 7.5 (0.1M phosphate) for 9.0 hours; pH 9.0 (0.1M veronal) for 12 and 24 hours; and pH 10.0 (0.1M phosphate-borate) for 9.0 hours. The experiments on cellulose-acetate matrix were run for 45 minutes at pH 8.6 (0.05M veronal) and pH 8.6-9.1 (discontinuous buffer) under a field of 275 volts (5 ma.). Paper strips containing a sample of 1% starch solution were used to establish the extent of migration of solvent in the L.K.B. apparatus.

When the electrophoretic run was complete, the paper strips were hung to dry in the air. When dry, the strips were immersed in a bromphenol blue solution (1 g of the indicator per liter of ethanol saturated with mercuric chloride). The strips were washed with 1% acetic acid solution and the dye was fixed by immersing in an acetate buffer (50 ml. of glacial acetic acid and 4.0 g of sodium acetate in 1 liter of water) and drying the strips in an oven at 120°.

The cellulose-acetate strips were stained with light green SF dye and fixed with dilute trichloroacetic acid.

8. Analytical Ultracentrifugation

Two purified enzyme preparations were combined and adjusted to 60 per cent salt saturation with ammonium sulfate. The cloudy suspension was centrifuged at 16,000 rpm and the supernatant was discarded. The precipitate was taken in 0.5 ml. of water and the mixture was centrifuged again at 16,000 rpm to sediment the insoluble matter. The supernatant was decanted into a cellophane tubing. The insoluble matter was suspended in 0.3 ml. of water, the suspension was centrifuged, and the clear supernatant was added to the cellophane tubing. Then, the enzyme preparation was dialysed in 0.1M sodium chloride solution (2 liters) for 6 hours. The resulting enzyme solution contained 5.93 mg of protein per ml. (specific activity 153,000).

This enzyme preparation was examined in the Spinco Model E analytical centrifuge (83). The sedimentation velocity experiment was run at 42,040 rpm, and photographs of the sedimenting boundaries were taken at intervals of 8 minutes. For the determination of the molecular weight of the major component by the Archibald method, the enzyme preparation was subjected to 10,589 rpm and photographs of the resulting boundary were taken at intervals of 16 minutes ($\theta = 75^{\circ}$). The enzyme solution was transferred to the synthetic boundary type cell, the synthetic boundary was photographed, and then, it was subjected to 59,780 rpm (the sedimentation pattern photographed at intervals of 8 minutes). Measurements on the sedimentation patterns were carried out with a microcomparator.

9. Stability of Enzyme vs. pH

One ml. of enzyme solution (5,400 phenolphthalein units per ml.,

spec. act. 108,000) was diluted to 5.0 ml. with water. Two tenths ml. of this dilution were added to 0.2 ml. of the appropriate buffer: pH 2.2 and 3.0 (0.1M glycine-HCl); pH 3.8, 4.3, 4.9, and 5.7 (0.1M acetate); pH 8.1 and 9.1 (0.1M tris-HCl); and pH 10.4 (0.1M glycine-NaOH). The mixtures were allowed to stand at room temperature (23°) for four hours at the end of which the pH of the mixtures was readjusted to pH 3.8 by adding 3.6 ml. of 0.1M acetate buffer. Five tenths ml. of phenolphthalein glucuronide solution were added to 1.0 ml. aliquots of the resulting mixtures, and the hydrolysis was allowed to proceed for 30 minutes at 37°. The determinations were carried out also with an enzyme dilution to which bovine serum albumin was added (100 μg per ml.)

10. Effect of Temperature

An assay mixture consisting of 0.5 ml. of 0.1M acetate buffer, pH 4.5; 0.5 ml. of 0.0015M phenolphthalein glucuronide solution; 0.4 ml. of water; and 0.1 ml. of enzyme dilution (0.006 mg of protein per ml., specific activity 80,000) was incubated for 30 minutes at temperatures of 10°, 25°, and 37°. The assay mixture was incubated for only 5 minutes at 47°.

11. Inhibition by Cations

A purified enzyme solution (0.76 mg per ml., spec. act. 112,000) was diluted 1:250 for the experiments. No albumin was added to this dilution. The assay mixtures consisted of 0.3 ml. of serial dilutions of 0.01M cation solution; 0.9 ml. of 0.1M acetate buffer, pH 4.5; 0.2 ml. of the enzyme dilution; and 0.1 ml. of 0.0075M phenolphthalein glucuronide solution. The mixtures were incubated at 37° for 30 minutes.

The effect of silver, cupric, mercuric, calcium, magnesium, manganese and zinc ions was investigated.

12. Inhibition by Sulfhydryl-Group Reagents

(a) N-Ethyl maleimide. — A 0.02M solution of this inhibitor was prepared in 0.1M acetate buffer, pH 4.5. Aliquots of 0, 0.075, 0.30, and 0.75 ml. of this solution were placed in test tubes and the volumes were brought to 1.2 ml. with additional acetate buffer. Two tenths ml. of the enzyme dilution (3 μg of protein per ml., spec. act. 112,000) were added to each test tube and the tubes were allowed to stand at room temperature for 90 minutes. Then, 0.1 ml. of 0.0075M phenolphthalein glucuronide solution was added and the mixtures were incubated for 30 minutes at 37°.

(b) p-Hydroxy mercuribenzoate. — A 0.0001M solution of this substance was prepared in 0.1M acetate buffer, pH 4.5. Aliquots of 0, 0.15, 0.30, 0.75, and 1.2 ml. of this solution were placed in test tubes and the volumes adjusted to 1.2 ml. with additional acetate buffer. Two tenths ml. of the enzyme dilution (same as in a) were put in each test tube and the mixtures were allowed to stand at room temperature for 30 minutes. Then, 0.1 ml. of 0.0075M phenolphthalein glucuronide solution was added and the mixtures were incubated for 30 minutes at 37°.

To check the effect of this agent at pH 8.1, a 0.0001M solution was prepared in 0.1M tris-HCl buffer, pH 8.1. Two tenths ml. of this solution were added to 0.2 ml. of enzyme dilution (1,080 units per ml., spec. act. 108,000) and the mixtures were allowed to stand 30 minutes at room temperature. At the end of the 30 minutes, 3.6 ml. of 0.1M

acetate buffer, pH 3.8, were added to each test tube. One ml. aliquots of the resulting mixtures, combined with 0.5 ml. of 0.0015M phenolphthalein glucuronide solution, were incubated for 30 minutes at 37°.

III. RESULTS AND DISCUSSION

1. Purification of the Enzyme

Initially, the snails were killed and the digestive tract was converted to acetone-powder. The acetone-powder retained full activity of the enzyme and this could be extracted by suspending the powder in 20 per cent saturated ammonium sulfate. The preparation of acetone-powder was discontinued because during the fractional elution of the enzyme from DEAE-cellulose two consecutive fractions were obtained with identical specific activity; this did not happen when the digestive tracts of the snails were homogenized in dilute ammonium sulfate solution. Otherwise, steps 1-5 of the procedure, when starting with acetone-powder, yielded the same degree of purification as when starting with the homogenate of fresh tissues.

It was found convenient, in order to work with a cleaner extract, always to centrifuge the homogenate, remove the black insoluble matter, and then heat the extract to 70-74°. Nevertheless, heating of the homogenate gave the same results as far as increasing the specific activity was concerned. The heat denaturation step always yielded very good recovery of the glucuronidase activity (Table I). The extract, after the heat denaturation step, retained a deep red-brown color. The bulk of the colored material was removed in Step 3 when the proteins were precipitated with ammonium sulfate and the colored supernatant was discarded.

Table I. Effect of heating the enzyme extract at 70-74°

Number of Snails Killed	Spec. Act. of Extract		Per cent Activity
	Before Heating	After Heating	
200*	300	600	87
81	360	1,030	85
150	430	1,100	89
115	540	1,260	91
140	400	933	91
200	220	580	81
200	220	590	80
200	200	618	74
185	220	700	86
105**	200	730	99
500**	242	750	95

* Enzyme was extracted from the acetone-powder of snails.

**The denatured proteins were centrifuged out and extracted once before discarded.

Application of steps 1-4 of the procedure gave about 12-fold purification of the material in the first extract (Table II). The values obtained with one of the batches of snails suggest that the snails in different batches may show a great difference in their enzyme content. The purification of the enzyme from acetone-powder was included in the table for comparison.

The crude enzyme preparations destroyed the cellophane tubing during pervaporation and during dialysis in phosphate buffer, pH 7.0. However, it was found safe to dialyse these preparations in ammonium

Table II. Extent of enzyme purification given by steps 1-4.

Snails in Batch	mg Prot.	Spec. Act.	Fold-Purif.	Per cent Activity
200*	340	4,500	15	69
486	1,478	3,150	7.6	63
785	2,365	2,610	12	53
105	625	2,560	13	75
500	1,000	2,482	11	57

*Enzyme was extracted from the acetone-powder of snails.

Table III. Fractional elution of the enzyme from DEAE-resins.

Resin*	Fraction Placed in Column		DEAE-Eluate	
	mg Prot.	Spec. Act.	Spec. Act.	Per cent Act.
4 g of (a)	130	2,600	71,000	23
22 g of (b)	620	3,200	110,000	16
22 g of (b)	450	2,600	91,000	12
22 g of (b)	450	2,360	91,000	17
22 g of (b)	450	2,570	87,000	12
22 g of (c)	570	2,870	47,000**	23
22 g of (c)	312	2,560	60,000**	32
22 g of (c)	500	2,480	73,000	13
22 g of (c)	250	2,480	100,000	21
22 g of (c)	250	2,480	95,000	24

* Resins: (a) DEAE-Sephadex A50 (3.9 meq/g); (b) DEAE-cellulose (0.78 meq/g); (c) DEAE-cellulose (062 meq/g).

** The enzyme was eluted during the washing of the column with 0.005M buffer.

sulfate solutions. This effect was attributed to the presence of cellulase activity in the crude preparations. When it was necessary to dialyse crude fractions in 0.005M phosphate buffer, the cellophane tubings were changed at intervals of about two hours.

The fractional elution of the enzyme from DEAE-cellulose gave about 35-fold purification of the crude fractions placed in the column (Table III). Usually, very little of the glucuronidase was eluted with the 0.005M buffer, but when the columns were overloaded the bulk of the activity was eluted during the washing-elution. The elution with 0.01M buffer gave a protein peak coinciding with a sharp peak of enzyme activity (Figure 1). The fractions corresponding to both sides of the peak were worth saving since they contained considerable amounts of the enzyme, although of less purity than the fractions corresponding to the top of the peak. Re-elution of these fractions from DEAE-cellulose usually yielded eluates of high specific activity. DEAE-cellulose (0.78 meq/g) was the resin most effective for the purification of the enzyme; DEAE-cellulose (0.62 meq/g) was effective when the column was charged with small crude enzyme fractions. DEAE-Solk Floc and DEAE-Sephadex A50 were even less effective, and Sephadex G-75 gave no purification of the crude fractions at all.

Precipitation of the DEAE-eluates with ammonium sulfate, besides concentrating the proteins, always led to an increase in the purity of the enzyme preparation (Table IV). The increase in specific activity given by Step 6 could also be obtained by placing the eluates in a DEAE-cellulose column for a second elution. However, the ammonium sulfate precipitation gave a much better recovery of the activity. Preparations with specific activity higher than 110,000 were not affected by the ammonium sulfate precipitation.

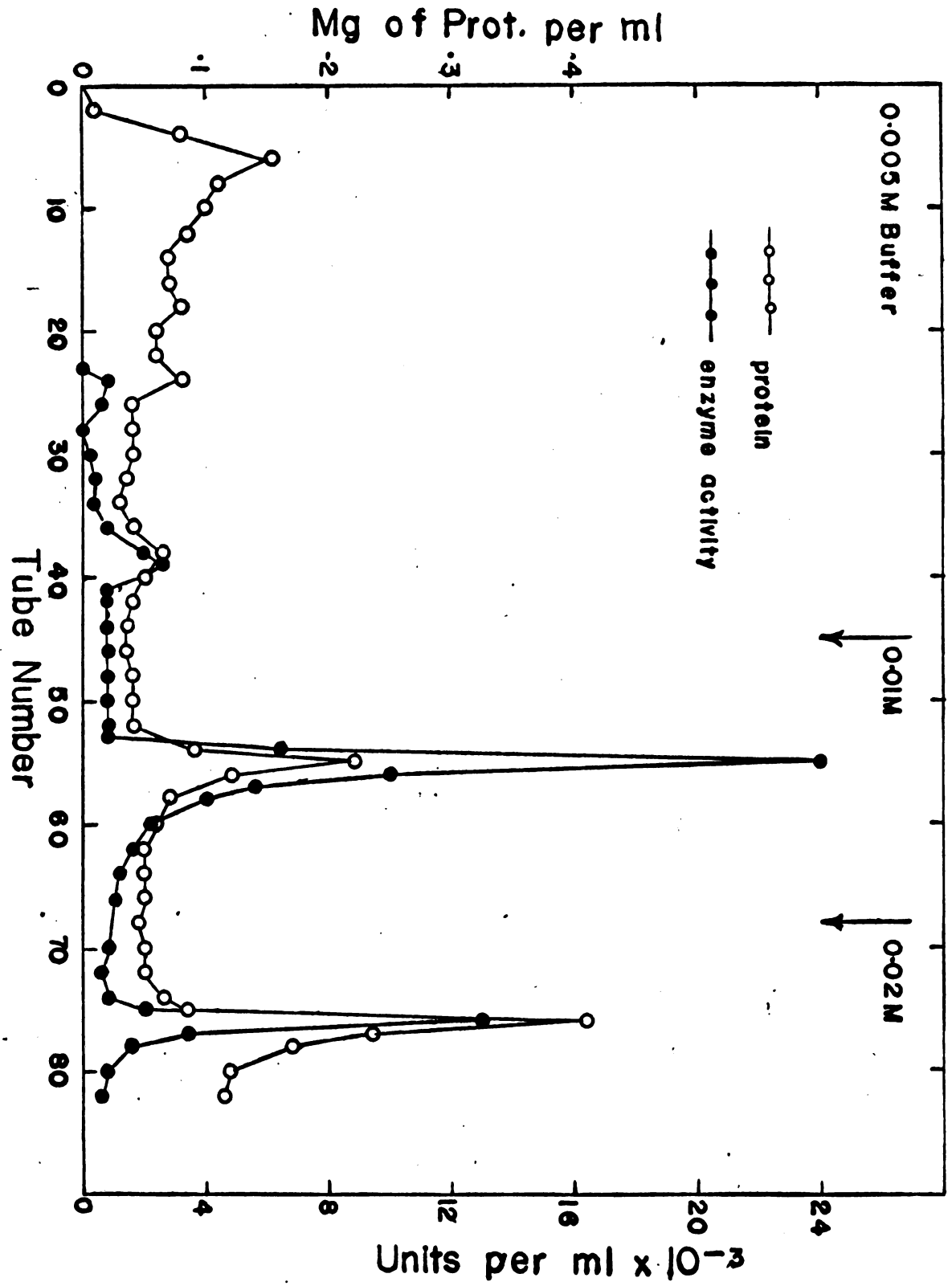

Figure 1. Fractional elution of β-D-glucuronidase from DEAE-cellulose (0.78 meq per g). A fraction of 620 mg of protein (apec. Act. 3,200) was placed in the column.

Table IV. Effect of precipitating the DEAE-eluates with ammonium sulfate

DEAE-Eluate		Water-Soluble Precipitate		Per cent
mg Prot.	Spec. Act.	mg Prot.	Spec. Act.	Activity
9.90	55,000	4.90	100,000	90
8.10	86,000	5.35	122,000	94
15.8	42,000	10.1	57,000	88
7.56	54,000	5.00	77,000	95
1.46	124,000	1.28	123,000	90
22.2	113,000	18.7	114,000	85
6.94	92,000	4.50	118,000	83

Table V summarizes a typical purification procedure. The whole procedure gave a 500-fold purification of the material in the first extract. The purified preparation was colorless and amorphous. It was free of arylsulfatase activity as evidenced by the fact that 9 μg of protein from the enzyme preparation did not cause hydrolysis of nitrochatecol sulfate and p-nitrophenol sulfate after one hour at 37° and pH 5.5 (84). The preparation was probably free of cellulase activity also.

An attempt to crystallize the enzyme was made by subjecting a combination of various purified preparations (18.7 mg prot. in 5.5 ml.; spec. act. 114,000) to slowly increasing ammonium sulfate concentration. By this slow increasing of salt concentration, the preparation required less than 35 per cent saturation to precipitate. The protein separated as a white, floculent precipitate. The suspension was centrifuged at 7,000 rpm to sediment the precipitate. It appeared amorphous under the microscope, but it exhibited the same specific activity as

Table V. Purification of β-D-glucuronidase from the aquatic snail, Ampullaria cupina.

Step	Fract.	mg Prot.	Enz. Act.	Spec. Act.	Per cent Yield Act.
1	I	18,800	4,524,000	242	100
2	III	5,720	4,298,000	750	95
3	VII	2,040	3,908,000	1,920	86
4	XI	1,000	2,482,000	2,480	57
5	DEAE-eluate	6.94	639,000	92,000	14.1
6	Sol. ppt.	4.50	530,000	118,000	11.7

500 snails (Mainly small)

did the preparation placed in the cellophane tubing. The supernatant showed a specific activity of only 37,000. The ratio of the absorbance at 280 mμ to the absorbance at 260 mμ for the precipitated protein dissolved in water (50 μg/ml.) was 1.25. This was a rather low value for a protein and suggested the presence of contaminating materials in the preparation. The precipitate was redissolved in water and centrifuged to remove the insoluble material. For another four times, the enzyme preparation was subjected to increasing salt concentrations. The rate of addition of the salt solution was made slower every time; the last addition running for nearly two weeks. Every time a precipitate appeared, samples were observed in the microscope and they were found to be amorphous. The precipitates were redissolved by dialysis in distilled water and any insoluble material in the preparation was removed by centrifugation. These five precipitations did not cause the crystallization of the enzyme, but they improved the purity of the enzyme since after the treatment the specific activity of the preparation was 168,000. The activity of this individual preparation was higher than any previously reported for non-mammalian preparations (3, 39, 40) and second only to the rat preputial gland preparation (38). In phthalate buffer, pH 3.1, the activity of this preparation was 420,000 Fishman Units per mg. of protein (Figure 5).

2. Test of Homogeneity (Zone Electrophoresis)

The most active fraction obtained (specific activity 168,000) was found electrophoretically homogeneous on paper and on cellulose-acetate. The enzyme was not stable at pH values below 4.5, but it was observed that the migration was toward the cathode. The enzyme appeared to have

the isoelectric point near pH 4.5. The migration on paper as a single spot, at different pH values, was as shown in Table VI.

Table VI. Paper electrophoresis in L.K.B. apparatus.

Buffer, pH	Time (hrs.)	Distance Moved Toward Anode
0.1M acetate, pH 4.5	9.5	0.0 cm.
0.1M phosphate, pH 7.5	9.0	5.8 cm.
0.1M veronal, pH 9.0	12.0	5.6 cm.
0.1M veronal, pH 9.0	24.0	9.0 cm.
0.1M phos.-borate, pH 10	20.0	7.5 cm.

3. Analytical Ultracentrifugation

Since the Archibald method of molecular weight determination as well as the determination of the sedimentation coefficient do not require a homogeneous preparation, the enzyme solution with specific activity of 153,000 was sedimented in the analytical ultracentrifuge with the purpose of approximating these physical properties and also establishing the relative composition of the preparation. When the preparation was subjected to 42,040 or 59,780 rpm, it gave a sharp, symmetrical peak which was preceded and followed by very small peaks. Figure 2 shows the synthetic boundary (zero time) and the sedimentation pattern obtained at 59,780 rpm. The sedimentation coefficient of the major peak ($S_{20,w}$) was calculated to be 11 S. The value of the coefficient did not change with time for the entire run (72 minutes at 42,040 rpm). The area of the sedimentation pattern, corrected for radial dilution (85), for the major peak at top speed was accounted for as

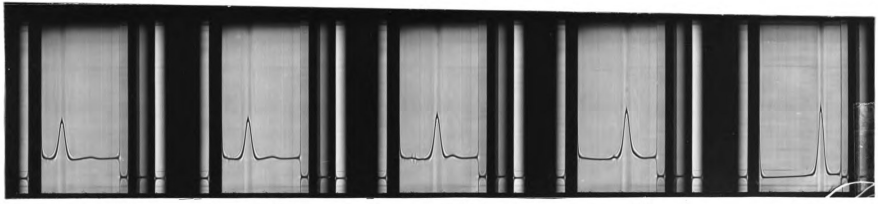

Figure 2. Sedimentation of an enzyme preparation with specific activity of 153,000 in the Analytical Ultracentrifuge at top speed (59,780 rpm). (a) synthetic boundary at zero time; (b) 8 minutes, (c) 16 minutes, (d) 24 minutes, and (e) 28 minutes pictures.

80 per cent of the synthetic boundary at zero time and at top speed (initial area). This was indication that the major component represented 80 per cent of the material in the enzyme preparation. This result suggests that the limiting value of the specific activity of the enzyme is 190,000 at pH 4.5 (acetatebuffer). During the approach to equilibrium experiment the preparation gave a boundary pattern which after 48 minutes began to look like a peak (Figure 3). Measurements for the computation of the molecular weight were taken from the 32, 64, and 112-minute photographs. Using the density of the sodium chloride solution (1.0025) and assuming a value for the specific volume of the protein (0.74 cc/g), the molecular weight was calculated to be 407,000 ± 10,000. A molecular weight as high as this supports the proposal that the inactivation of purified mammalian glucuronidase preparations on dilution is due to dissociation into inactive components (57).

4. Stability as Function of pH

Table VII. Effect of pH on the stability of the enzyme.

pH	Per cent Recovery	
	0 B.S. Alb./ml.	100 µg B.S.Alb./ml.
2.2	0	0
3.0	3	47
3.8	8	86
4.9	31	92
5.7	42	89
8.1	55	76
9.1	58	84
10.4	55	80

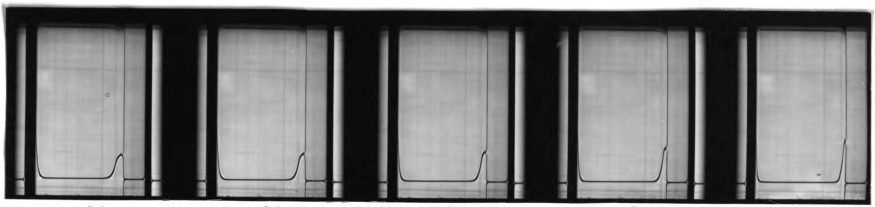

Figure 3. Sedimentation of the enzyme preparation (specific activity 153,000) at 10,589 rpm. (a) 16 minutes, (b) 32 minutes, (c) 48 minutes, (d) 64 minutes, (e) 80 minutes, (f) 96 minutes, (g) 112 minutes, and (h) 128 minutes, pictures; (i) and (j) synthetic boundary.

These results show that the enzyme from A. cupina resembles preparations from P. vulgata (51) and L. littorea (52) in stability when albumin is added to the dilutions. The activating effect by albumin is probably attributable to a stabilizing action. P. Bernfeld et al. (57) studied the inactivation of mammalian preparations on dilution and explained the phenomena as dissociation of the enzymic protein into inactive components. They explained the activation by albumin, deoxyribonucleic acid and other agents as a prevention of such dissociation. At 37°, the A. cupina enzyme was stable only at pH values above 4.5.

5. Effect of Enzyme Concentration

The hydrolysis of phenolphthalein glucuronide varied linearly with the amount of enzyme present in the assay mixture (Table VIII).

Table VIII. Effect of enzyme concentration

µg E. Prot./mixture	µg Phenolphthalein Prod. per hour
0.30	22
0.48	36
0.60	44
0.78	57
0.90	66
1.20	88

Assays at pH 4.5 (actate). B.S.Albumin in E. dilution: 100 µg/ml. Specific activity of enzyme prep.: 80,000.

6. Time Course for the Hydrolysis of Various Substrates

The enzymic hydrolysis of phenolphthalein-, 1-menthyl, pregnanediol, and p-nitrophenyl β-D- glucuronides as function of time is shown

in Figure 4. Without apparent reason, the enzyme preparation failed to hydrolyse the glycosidic bond of borneol β-D-glucuronide. It was observed, however, that when this substrate was put in solution effervescent occurred; this did not happen with any of the other substrates. The enzyme dilution was sufficiently strong for complete hydrolysis of phenolphthalein glucuronide in less than 45 minutes. After 90 minutes, the hydrolysis of pregnanediol glucuronide was nearly complete. Menthyl glucuronide was 61 per cent and p-nitrophenyl glucuronide 78 per cent hydrolysed after two hours of incubation. The hydrolysis of pregnanediol and phenolphthalein glucuronides appeared to be linear with time to nearly completion of reaction, but not the hydrolysis of the other two substrates. The little specificity exhibited by the present glucuronidase preparation towards the aglycon part of the substrate, is the usual behaviour of β-glucuronidases regardless of the source.

7. Effect of Temperature on the Enzyme Activity

Table IX. Effect of temperature on the β-glucuronidase activity.

Temperature (°C)	Velocity of Hydrolysis
10	0.29
25	1.6
37	4.4
48	9.4

Velocity in μ moles of S. split per min. per mg of protein.

From these results it was calculated that the energy of activation for the enzymic hydrolysis of phenolphthalein glucuronide is -16,000 cal.

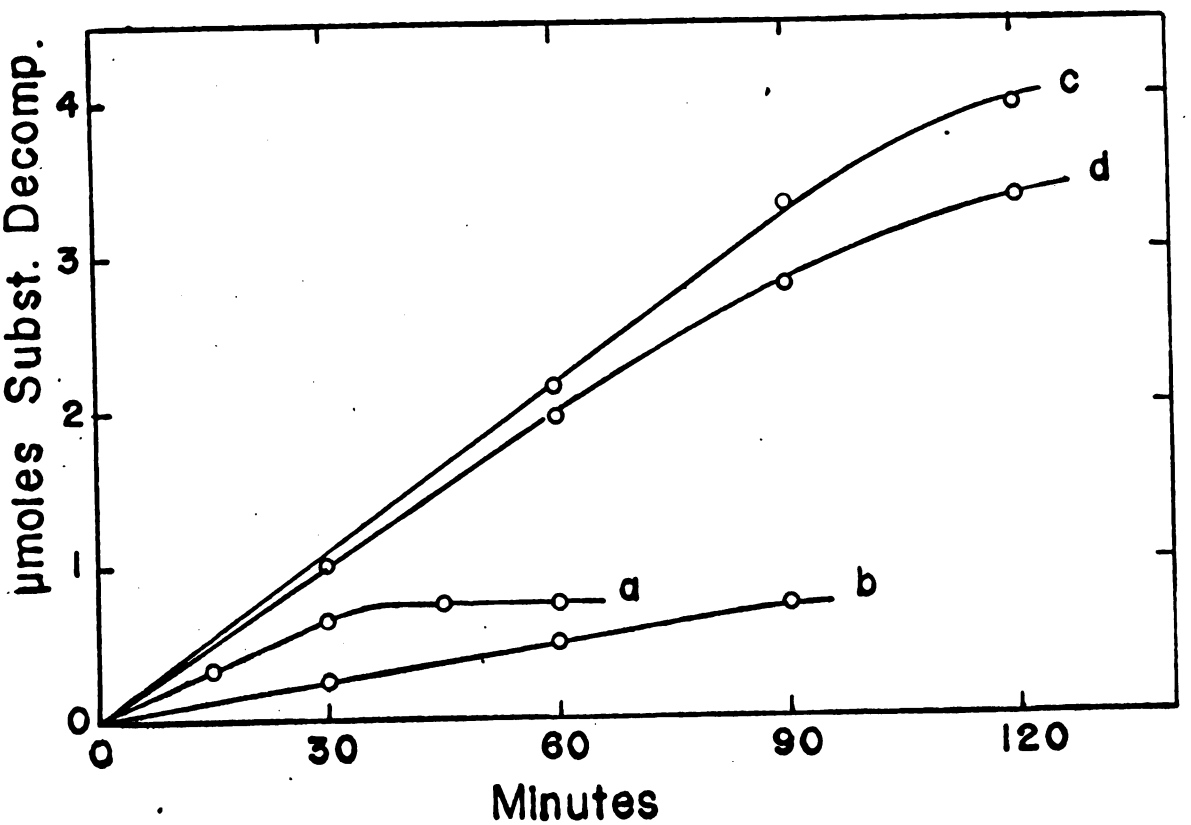

Figure 4. Time course for the hydrolysis of several substrates. All determinations were done with 0.1M phthalate buffer. An enzyme preparation with spec. act. of 138,000 was diluted to 1.23 µg of protein per ml. and 0.5 ml of this dilution used for the determinations. (a) 0.0005M phenolphthalein glucuronide at pH 3.6; (b) 0.0005M pregnanediol glucuronide at pH 4.5; (c) 0.0033M 1-menthyl glucuronide at pH 4.2; and (d) 0.0029M p-nitrophenyl glucuronide at pH 3.9.

per mole. This result is in the range of other values reported for mammalian (35, 54) or non-mammalian preparations (55).

8. Effect of pH (and Buffers) on the Enzyme Activity

Previous preparations of the β-glucuronidases from other sources have been always reported to have an optimum pH for activity (3, 4). Figure 5 shows the effect of pH on the velocity of hydrolysis, catalysed by the enzyme from A. cupina, of phenolphthalein glucuronide in acetate, citrate-phosphate, and phthalate buffers. The enzyme exhibits a sigmoid pH-velocity curve in which the activity approaches a maximum with decreasing pH. The activity was affected by the nature of the buffering ions, being equal in acetate and citrate-phosphate buffers but nearly twice in phthalate buffer at very acidic pH values. At pH values above 5.0 the activity appeared to be equal in acetate and phthalate buffers; it was rather smaller in citrate-phosphate buffer.

When the action of an enzyme is studied, it is always assumed that the enzyme combines with its substrate to form an unstable complex which then may break down to products. If the maximum celocity varies with pH, this indicates that the enzyme-substrate complex ionizes or that its breakdown is subjected to acid or base catalysis. If a bell-shaped pH-velocity curve is obtained experimentally, it is assumed that there are two groups in the enzymatic site which have a total effect on the kinetics (86). The bell-shaped pH-velocity curve is considered to be a combination of the dissociation curves of the groups which may be of either the acidic or the basic types. If the pK values of the groups are sufficiently apart, these can be read from the inflection points on both sides of the bell-shaped curve.

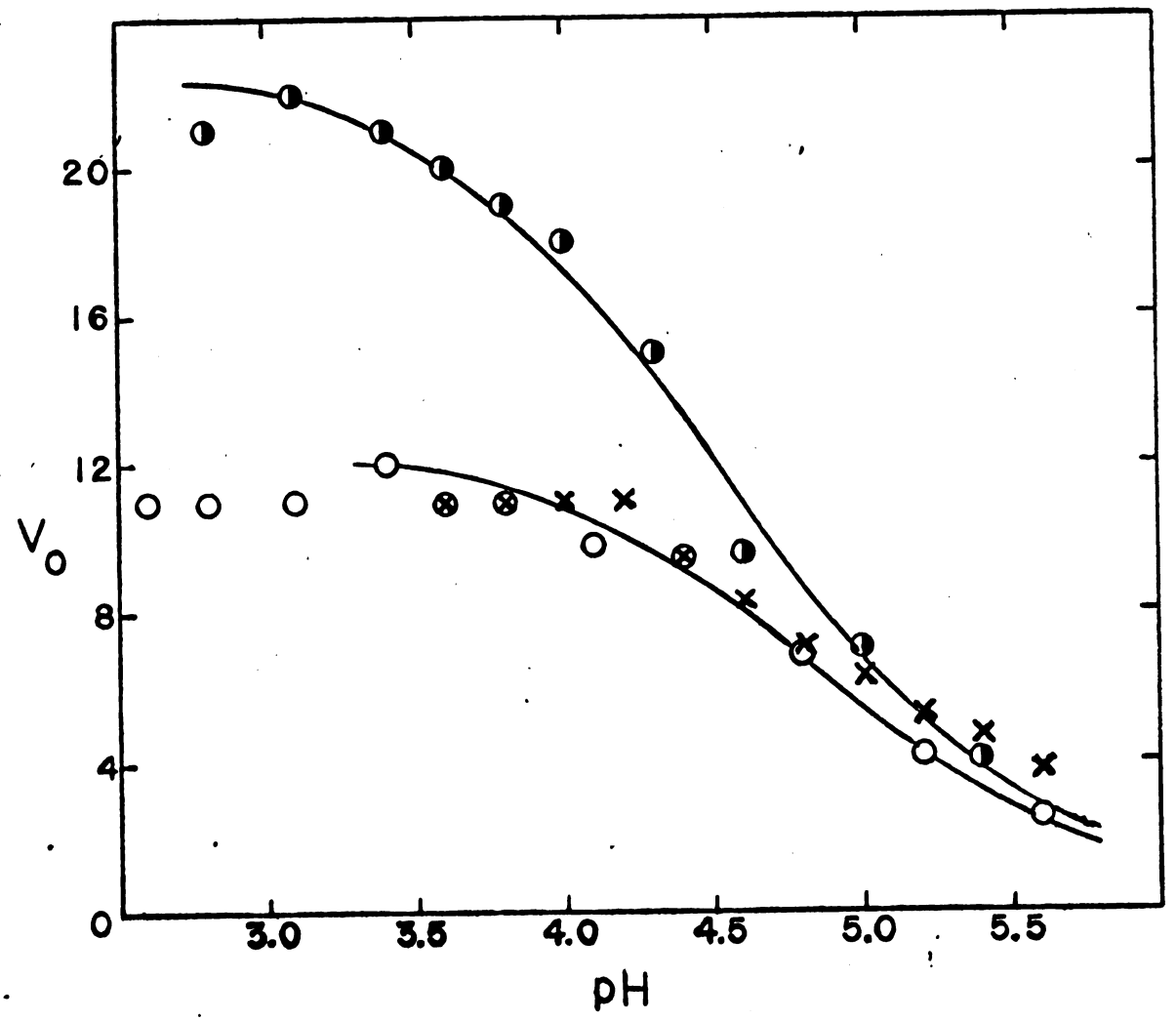

Figure 5. Effect of pH (and buffers) on the enzyme activity. 0.0005M phenolphthalein glucuronide hydrolyzed by the enzyme preparation with specific activity of 168,000. ◑ , with 0.1M phthalate buffer; X, with 0.1M acetate buffer; and ○ , with 0.1M citrate-phosphate buffer.

The velocity of the enzyme catalyzed hydrolysis of the phenolphthalein, 1-menthyl, and p-nitrophenyl β-D-glucuronides was determined at various pH values in phthalate buffer. The results, as shown in Figure 6, were in agreement with the fact that the nature of the aglycon has little effect on the ionization of the glucuronides (49). The pH-velocity curve for the hydrolysis of glucuronides exhibits the shape of the dissociation curve for a group with an approximate pK of 4.7. These results suggest that a carboxyl group of the enzyme is involved in the catalytic process that splits the glucuronosyl-O bond.

9. Effect of Substrate Concentration

The initial velocity of the hydrolytic reaction was determined for various concentrations of phenolphthalein glucuronide and p-nitrophenyl glucuronide. The velocity was determined from the amount of aglycon liberated in 1 to 3 minutes of incubation at 37°. The rate of hydrolysis of p-nitrophenyl glucuronide was maximum at 0.003M concentration of the substrate. For phenolphthalein glucuronide, the rate was maximum at 0.0005M substrate concentration, and it was inhibited by higher concentrations. The determinations were made in acetate and phthalate buffers for the phenolphthalein glucuronide and in phthalate buffer only for p-nitrophenyl glucuronide.

The results were analysed by the graphical method of Lineweaver and Burk (87). The effect of phenolphthalein glucuronide concentration on the enzyme activity is shown in Figure 7; the Michaelis constant was calculated to be 4.6 μM at pH 3.4 (phthalate). In acetate buffer, at pH 3.6, the value was 11 μM. Figure 8 shows that effect of p-nitrophenyl glucuronide concentration on the enzyme activity at pH 3.6 (phthalate). The Michaelis constant was calculated to be 60 μM.

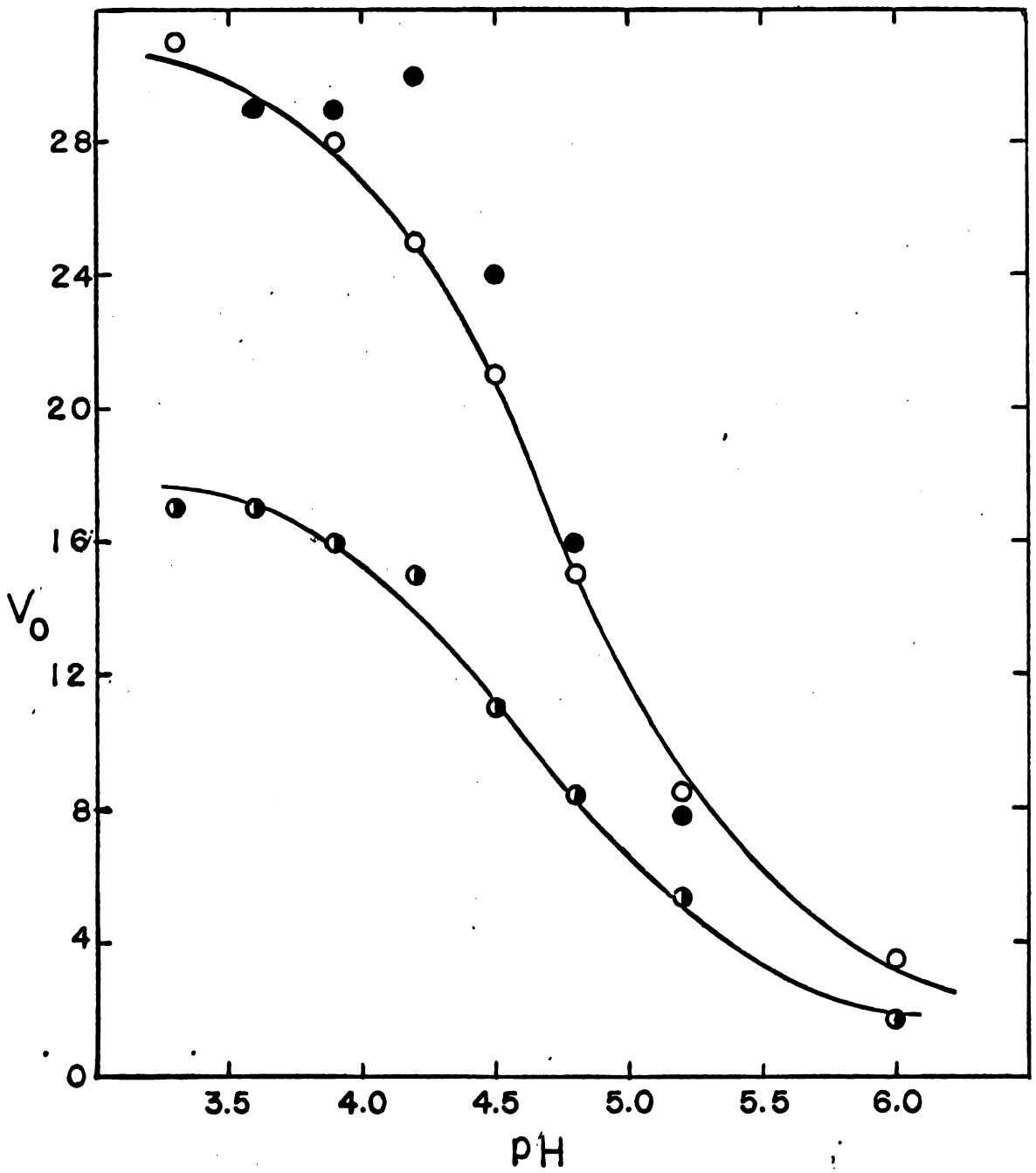

Figure 6. Effect of pH on the enzymic hydrolysis of various substrates. All determinations were done with 0.1M phthalate buffer. The enzyme preparation with specific activity of 138,000 was diluted to 2.5 µg of protein per ml. ● , 0.0033M l-menthyl glucuronide; O , 0.0029M p-nitrophenyl glucuronide; and ◑ , 0.0004M phenolphthalein glucuronide.

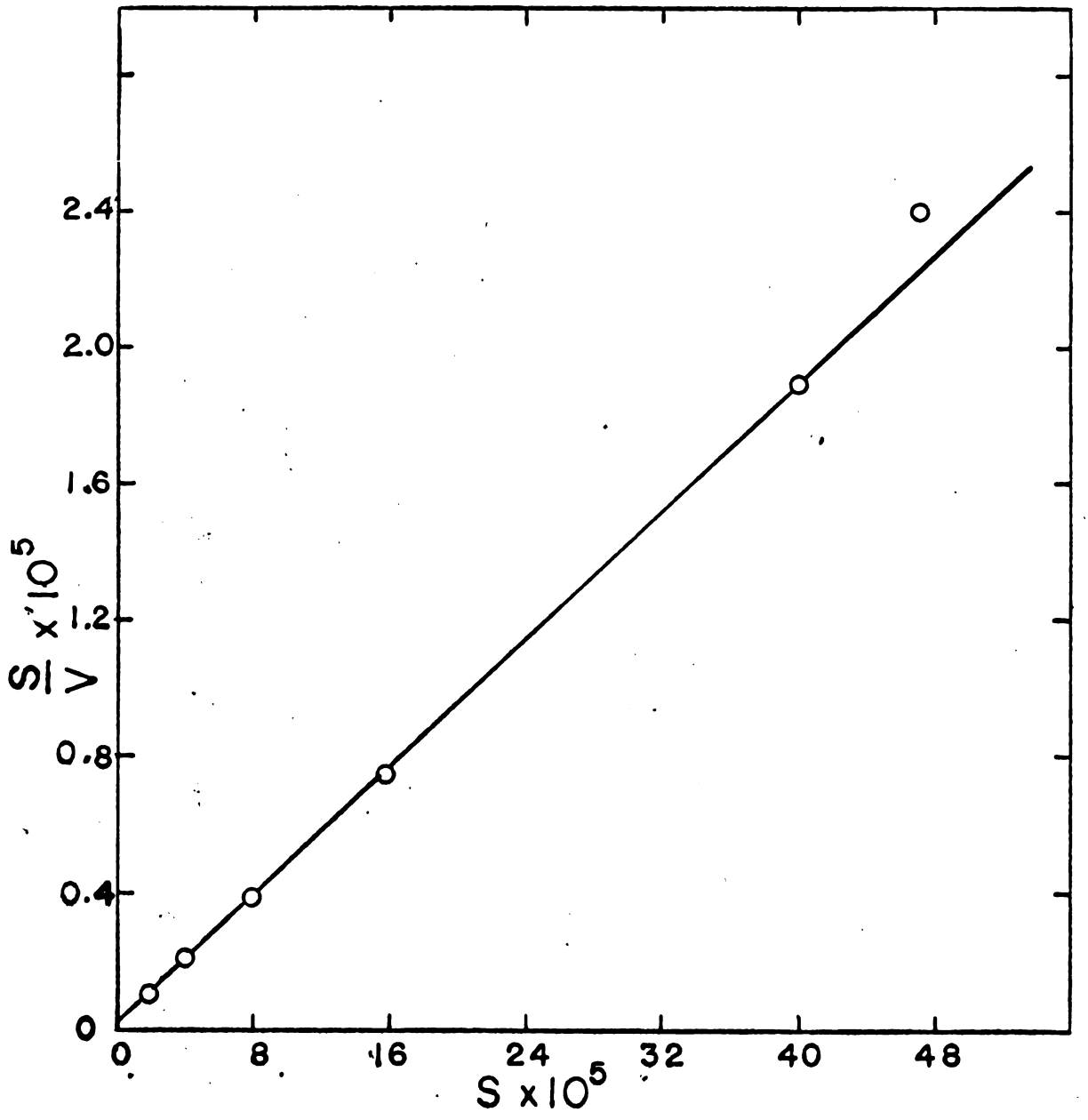

Figure 7. Effect of phenolphthalein glucuronide concentration on the enzyme preparation with specific activity of 168,000 was diluted to 0.58 μg of protein per ml. K_m was calculated to be 4.6 μM.

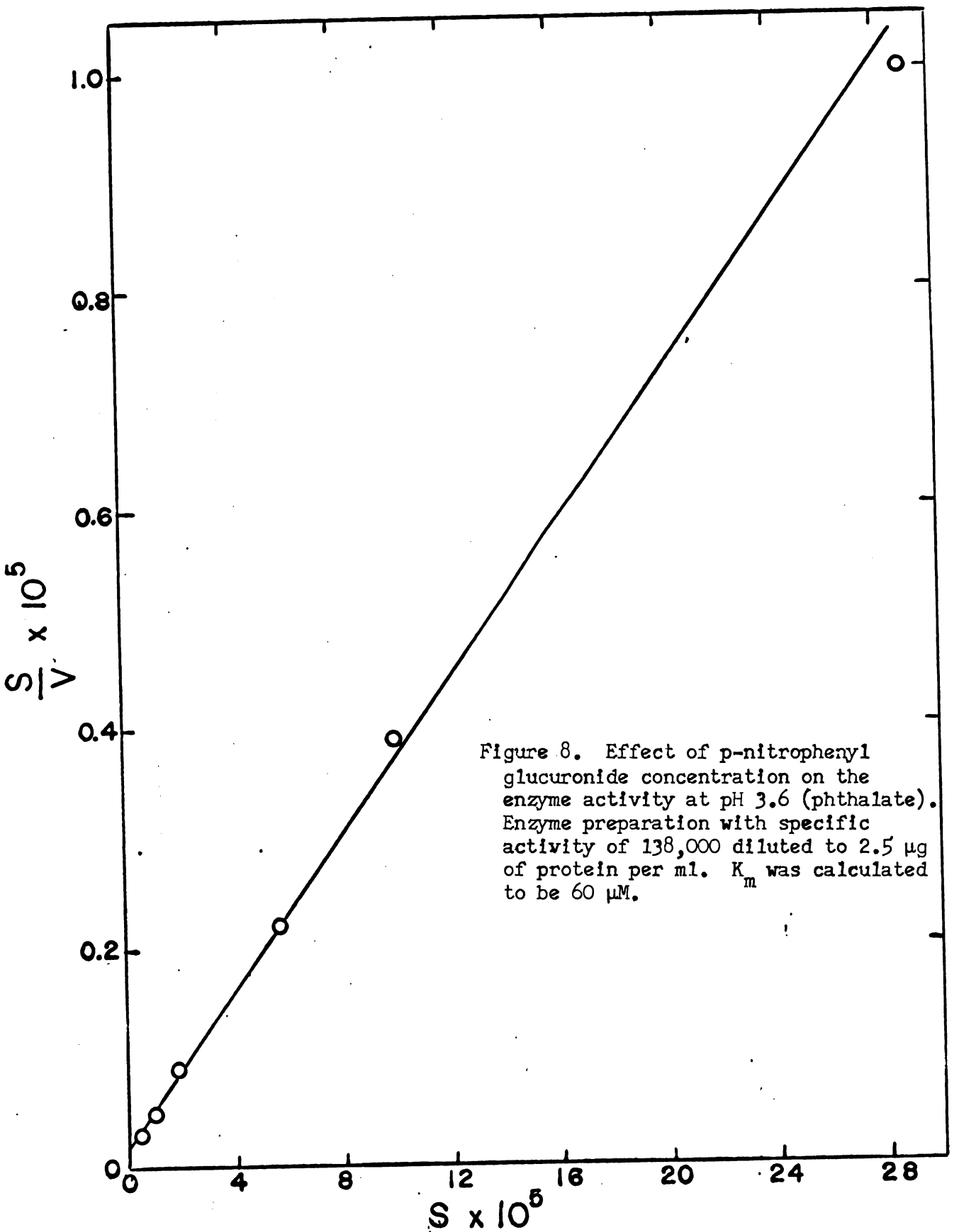

Figure 8. Effect of p-nitrophenyl glucuronide concentration on the enzyme activity at pH 3.6 (phthalate). Enzyme preparation with specific activity of 138,000 diluted to 2.5 µg of protein per ml. K_m was calculated to be 60 µM.

Dixon (88) has studied the influence of pH on the affinities of enzymes for their substrates. It was found that a plot of pK_m (negative logarithm of the Michaelis constant) versus pH would give valuable information of three kinds:

(a) on the nature of the enzyme-substrate link, deduced mainly from the slope of the curve;

(b) on the nature of the substrate-binding groups of the enzyme, deduced from the pK values as determined from the position of the discontinuities of the curve; caution needs to be exercised here (89);

(c) on the nature of the activation process, deduced from the ionizations of the enzyme-substrate complex. It was of interest to find out how much information could provide the effect of pH on the Michaelis constant for the glucuronidase catalyzed hydrolysis of phenolphthalein and p-nitrophenyl glucuronides.

The variation of enzyme activity with substrate concentration at various pH values was determined for phenolphthalein and p-nitrophenyl glucuronides. The Michaelis constant for phenolphthalein glucuronide varied with pH (phthalate buffer) from 4.6 μM at pH values below 4.0 to 17 μM at pH values above 5.0, and the variation was not linear. In acetate buffer, the variation was from 11 μM to 20 μM. However, the constant for p-nitrophenyl glucuronide was independent of the pH. Plots of pK_m versus pH are shown in Figure 9. The bending in the curve about pH 4.0 (concave downward) would be due to either a group in the free enzyme or a group in the substrate. Since this bending did not appear with p-nitrophenyl glucuronide as the substrate, it is concluded that it is due to a group in the substrate (probably the carboxyl group in the phenolphthalein moiety). The fact that the K_m for p-nitrophenyl

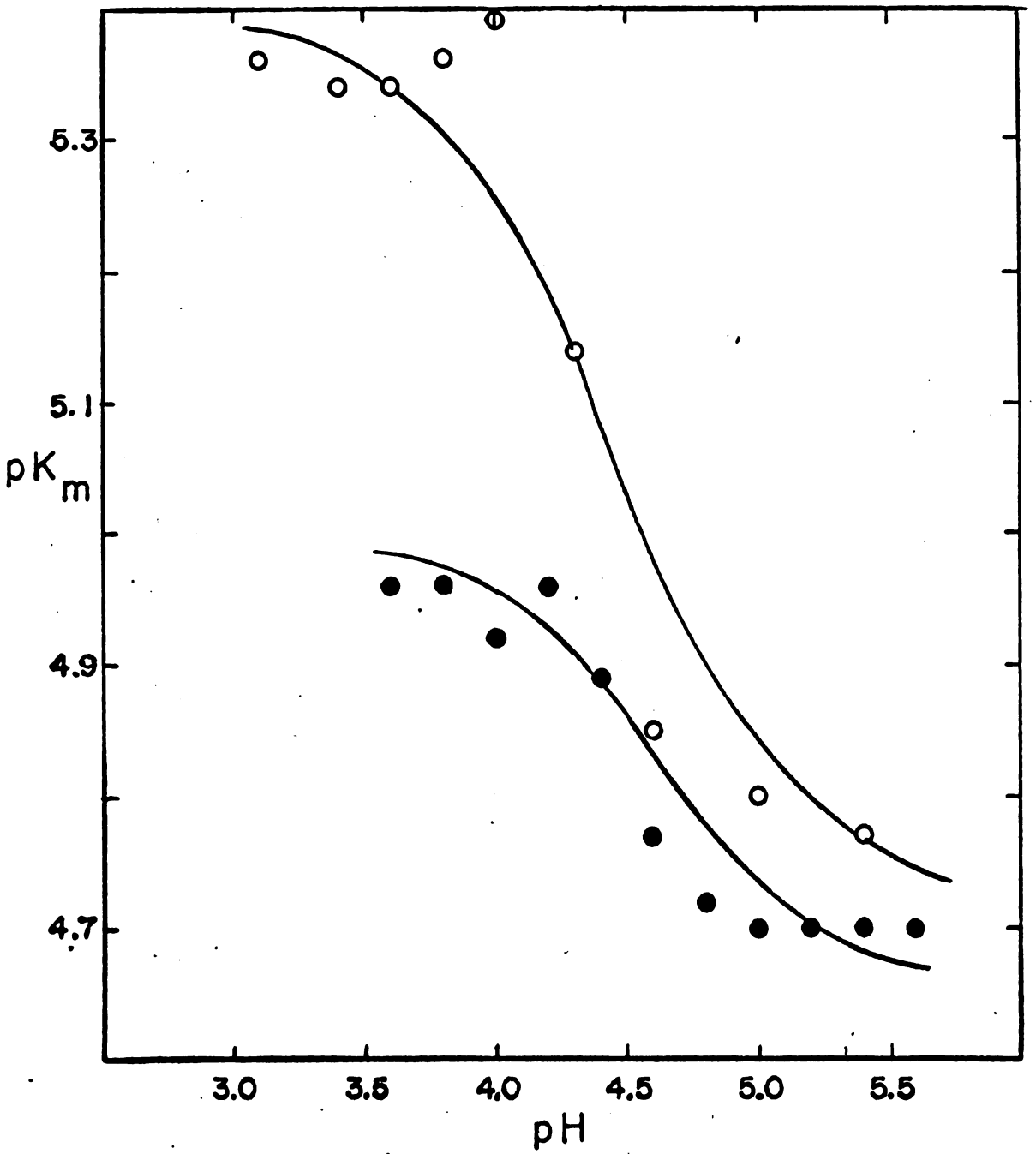

Figure 9. Effect of pH on the K_m for the β-D-glucuronidase catalyzed hydrolysis of phenolphthalein glucuronide. Enzyme preparation with specific activity of 168,000. O , determinations made in phthalate buffer; ● , determinations made in acetate buffer.

glucuronide does not depend on the pH would be explained either by a non-ionic interaction of substrate and enzyme or by an ionic one. Assuming that the binding is by ionic interaction, the groups involved probably would be an ammonium group in the enzyme and the carboxyl group of the substrate. Interaction between these groups should be pH-independent in the pH-region studied. It is possible that when phenolphthalein glucuronide is the substrate, a secondary interaction of the aglycon with the enzyme causes the K_m to be pH-dependent.

Linear variation of pK_m with pH (pH 3.0 to 5.5), with slope equal to -2.4, was observed for the enzyme preparation from the limpet Cellana tramoserica (56). Phenolphthalein glucuronide was the substrate.

10. Inhibition

The effects of silver, cupric and mercuric ions on the enzyme activity is shown in Table X. Mercuric ion was inhibitory at all concentrations while cupric and silver ions required concentrations higher than 2×10^{-4}M for effective inhibition. Low cupric and silver ions concentrations caused slight activation. A similar behaviour was reported for a mammalian preparation (63). Calcium, magnesium, manganese, and zinc ions had no effect on the enzyme activity. Dialysis in 0.01M versene solution caused no inhibition.

The sulfhydryl group reagents N-ethyl maleimide and p-chloromercuribenzoate failed to inhibit the enzyme; N-ethyl maleimide at pH 4.5 and p-chloromercuribenzoate at pH 4.5 and pH 8.0. These results appear to rule out the possibility of a sulfhydryl group being connected with the enzyme activity.

Table X. Inhibition by heavy cations.

Ion Conc. (M)	µg Aglycon per hour per ml. E. Dil.		
	Mercuric	Cupric	Silver
0	306	306	306
2×10^{-7}	294	323	323
2×10^{-6}	247	323	340
2×10^{-5}	59	323	370
2×10^{-4}	6	258	276
2×10^{-3}	0	-	28

IV. SUMMARY

1. A ndw β-D-glucuronidase of high activity has been prepared from the digestive tract of the aquatic snail, Ampullaria cupina, by a simple procedure. This procedure involved (1) extraction with 20% saturated ammonium sulfate., (2) heat denaturation, (3) precipitation of proteins with 55% saturated ammonium sulfate, (4) fractional precipitation with ammonium sulfate, (5) fractional elution from DEAE-cellulose, and (6) ammonium sulfate precipitation. The procedure gave a 500-fold purification of the material in the first extract. The final product was colorless and amorphous. It was free of arylsulfatase activity. The specific activity of the product was 120,000.

2. After several reprecipitations with ammonium sulfate, intended to crystallize the enzyme, the preparation was still amorphous but its specific activity increased by 40%. This highly active fraction was electrophoretically homogeneous.

3. Data obtained in the analytical ultracentrifuge at top speed indicated that the limiting value for the specific activity of the enzyme is 190,000. The sedimentation coefficient of the enzyme was calculated to be 11 S and the molecular weight as 407,000.

4. The diluted purified enzyme was stable between pH 4.0 and pH 10.4 if albumin was added to the enzyme dilutions.

5. The enzyme activity increased 2.3-fold for every ten degrees increase in temperature.

6. The velocity of hydrolysis, at pH 3.5 (phthalate), of pregnanediol, phenolphthalein, l-menthyl, and p-nitropehnyl glucuronides was

7.5, 17, 30 and 30 μ moles of substrate decomposed per min. per mg of protein respectively when they were hydrolyzed by a preparation with a specific activity of 138,000.

7. The enzyme exhibited a sigmoid pH-activity curve with inflection point about pH 4.7. The activity of the enzyme in acetate buffer and citrate-phosphate buffer was about 55% of the activity in phthalate buffer.

8. The Michaelis constant for phenolphthalein glucuronide showed variation with pH; the constant for p-nitrophenyl glucuronide was pH independent.

9. The enzyme was not inhibited by sulfhydryl group reagents. Mercuric, silver and cupric ions inhibited the enzyme activity, but only mercuric ion did it markedly.

V. APPENDIX

1. Calculations for the Analytical Ultracentrifugation

(a) Calculation of the sedimentation coefficient. The sedimentation experiment ran for 72 minutes at 42,040 rpm. Photographs of the sedimentation pattern were taken every eight minutes ($\theta = 70^{\circ}$).

$$S = \frac{2.303}{w^2} \log \frac{X_M/X_m}{t}$$

$w^2 = 19.381379 \times 10^6$

X_M = distance from axis of rotation to maximum ordinate of sedimentation pattern

X_m = distance from axis of rotation to meniscus

Distance from axis of rotation to reference hole in the rotor = 120.289.

Data and Results:

Time (sec.)	D_M	D_m	X_M	X_M/X_m	S
960	10.112	7.462	130.402	1.02075	11.014
1,440	11.438	7.454	131.728	1.0311	10.972
1,920	12.806	7.462	133.096	1.0418	11.008
2,400	14.185	7.463	134.475	1.0526	11.023
2,880	15.565	7.460	135.855	1.0634	11.008
3,360	16.972	7.464	137.262	1.0745	11.035
3,840	18.392	7.462	138.682	1.0855	11.023
4,320	19.835	7.462	140.125	1.0969	11.014

D_M - distance from reference hole to maximum ordinate of the sedimentation pattern.

D_m - distance from reference hole to meniscus.

X_m - 120.289 + 7.461 = 127.75.

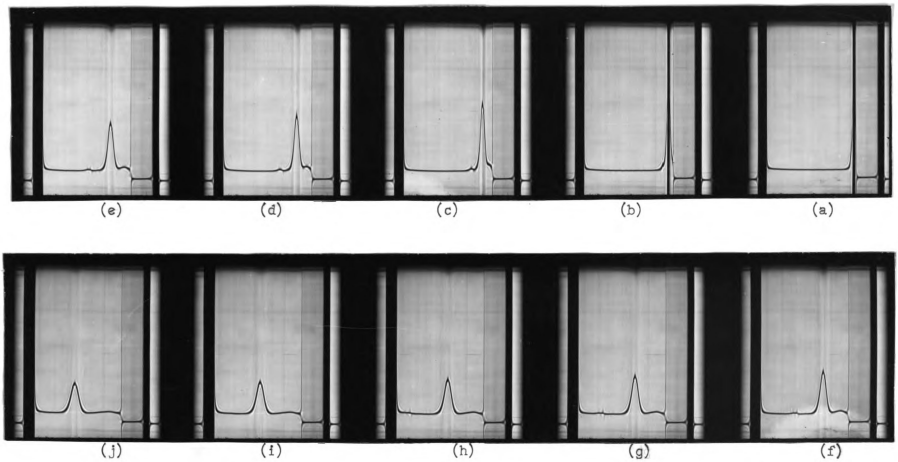

Figure 10. Sedimentation of the enzyme preparation at 42,040 rpm for the determination of the sedimentation coefficient. Rhotographs taken at (a) 4, (b) 8, (c) 16, (d) 24, (e) 32, (f) 40, (g) 48, (h) 56, (i) 64, and (j) 72 minutes.

(b) Calculation of the molecular weight. Determination of initial concentration (synthetic boundary at low speed; $\Theta = 75^{0}$).

$$c_0 = \frac{dx}{2.103} \sum Y_n$$

Data:

n	R_n(cm)	Y_n(cm)	n	R_n(cm)	Y_n(cm)
1	1.25	0.0470	18	1.42	1.3280
2	1.26	0.0550	19	1.43	1.2532
3	1.27	0.0704	20	1.44	1.1064
4	1.28	0.1014	21	1.45	0.9512
5	1.29	0.1394	22	1.46	0.8200
6	1.30	0.1938	23	1.47	0.6678
7	1.31	0.2588	24	1.48	0.5238
8	1.32	0.3580	25	1.49	0.4118
9	1.33	0.4592	26	1.50	0.3360
10	1.34	0.5624	27	1.51	0.2590
11	1.35	0.6850	28	1.52	0.1990
12	1.36	0.7878	29	1.53	0.1346
13	1.37	0.9050	30	1.54	0.0860
14	1.38	1.0142	31	1.55	0.0620
15	1.39	1.1060	32	1.56	0.0510
16	1.40	1.2118	33	1.57	0.0320
17	1.41	1.3290	34	1.58	0.0186

$$\sum Y_n = 17.5246$$

$$c_0 = \frac{(0.01)}{(2.103)} (17.5246)$$

$$= 0.08333$$

$$M_w = \frac{RT}{(1-\bar{V}\rho)w^2} \frac{(dc/dx)_m}{x_m c_m}$$

w^2 at 10,589 rev./min. = 1.229615×10^6

$\rho = 1.0025$ and $\bar{V} = 0.74$ cc/g

$T = 293^{0}$

$$M_w = 76,743 \frac{(dc/dx)_m}{x_m c_m}$$

Data at meniscus for 32 minutes centrifugation (10,589 rpm; $\theta = 75^0$)
($F = 2.103$; $\bar{V} = 0.74$ cc/g; $T = 293^0$; $w^2 = 1.229615 \times 10^6$; $\rho = 1.0025$)

n	R_n(cm)	Y_n(cm)	X_n	X_n^2
0	0.732	0.8616	12.719	161.773
1	0.742	0.8602	12.729	162.027
2	0.752	0.8572	12.739	162.282
3	0.762	0.8572	12.749	162.539
4	0.772	0.8566	12.759	162.792
5	0.782	0.8516	12.769	163.047
6	0.792	0.8388	12.779	163.303
7	0.802	0.8058	12.789	163.559
8	0.812	0.7606	12.799	163.814
9	0.822	0.6840	12.809	164.070
10	0.832	0.5916	12.819	164.327
11	0.842	0.5038	12.829	164.583
12	0.852	0.4308	12.839	164.840
13	0.862	0.3662	12.849	165.097
14	0.872	0.3104	12.859	165.354
15	0.882	0.2610	12.869	165.611
16	0.892	0.2194	12.879	165.869
17	0.902	0.1886	12.889	166.126
18	0.912	0.1498	12.899	166.384
19	0.922	0.1196	12.909	166.642
20	0.932	0.0974	12.919	166.901
21	0.942	0.0790	12.929	167.159
22	0.952	0.0628	12.939	167.418
23	0.962	0.0468	12.949	167.677
24	0.972	0.0376	12.959	167.936
25	0.982	0.0280	12.969	168.195
26	0.992	0.0140	12.979	168.454

$$\sum_n X_n^2 Y_n = 1921.0253$$

$$c_m = c_0 - \frac{dx}{(X_n^2)(2.103)} \sum_n X_n^2 Y_n$$

$$= 0.08333 - \frac{(0.01)(1921.0253)}{(161.773)(2.103)} = 0.02686$$

$$M_w = 76{,}743 \frac{0.8616}{(0.02686)(6.048)}$$

$$= 407{,}120$$

(c) Approximate composition of the enzyme solution. Concentration was corrected for radial dilution with:

$$C_o = F_x(X_r/x_m)^2 \Delta z \sum Y_j$$

Data for concentration at zero time (synthetic boundary at 59,780 rpm; zero time). $\theta = 70^o$

z	Y_j
570	0.536
575	1.254
580	3.932
585	8.922
590	13.962
595	16.894
600	11.180
605	5.796
610	2.560
615	0.926
620	0.300

$\sum Y_j = 66.262$
$\Delta z = 5$
$X_r = 160$
$x_m = 129.55$
$F_x = 0.02536$
$C_o = 12.815$

Data for concentration after 24 minutes centrifugation at top speed.

z	Y_j
690	0.310
695	0.756
700	1.780
705	3.812
710	6.442
715	8.758
720	10.038
725	8.500
730	5.688
735	3.294
740	1.734
745	0.728
750	0.340

$\sum Y_j = 52.180$
$C_o = 10.091$

2. Variation of Enzyme Activity with Substrate Concentration at Various pH Values

(a) For phenolphthalein glucuronide in acetate buffer.

Assay mixture: 0.5 ml. of 0.1M acetate buffer; 0.3 ml. of water; 0.2 ml. of enzyme dilution; and 0.5 ml. of phenolphthalein glucuronide dilution to give the desired final s. concentration.

Enzyme preparation with spec. act. of 168,000 was diluted to 1.56×10^{-3} mg of prot. per ml.

Results:

	v of hydrolysis at (S) x 10^5M conc. of substrate					
pH	1.58	3.15	9.46	23.7	47.3	118
3.6	7.1	9.5	11	11	11	
3.8	7.1	8.9	11	11	11	
4.0	6.0	8.3	9.5	10	11	
4.2	5.4	7.8	8.9	9.5	11	
4.4	4.8	7.1	7.7	8.9	9.5	
4.6		6.0	7.1	7.7	8.3	8.9
4.8		5.2	6.4	6.7	7.1	7.9
5.0		4.0	5.2	5.9	6.3	7.1
5.2		3.2	4.4	4.7	5.3	6.0
5.4		2.7	3.5	4.1	4.7	5.1
5.6		2.1	2.8	3.3	3.8	4.0

V = μ moles substrate decomposed per min. per mg of prot.

(b) For phenolphthalein glucuronide in phthalate buffer.

Assay mixture: 0.5 ml. of 0.1M phthalate buffer; 0.5 ml. of the enzyme dilution; and 0.5 ml. of substrate dilution.

Enzyme prep. with spec. act. of 168,000 was diluted to 5.8×10^{-4} mg of prot. per ml.

Results:

	v of hydrolysis at (S) x 10^5M conc. of substrate				
pH	1.89	3.94	7.88	15.8	47.3
3.1	18	20	21	22	21
3.4	17	19	20	21	20
3.6	17	18	20	20	19
3.8	16	17	18	19	19
4.0	15	17	18	18	19
4.3	11	13	14	15	15
4.6	6.0	6.5	8.5	9.1	9.6
5.0	4.3	5.1	5.7	6.4	7.1
5.4	2.0	2.8	3.2	3.6	4.1

(c) For p-nitrophenyl glucuronide in phthalate buffer.

Assay mixture: 0.5 ml. of 0.1M phthalate buffer, 0.5 ml. of p-nitrophenyl glucuronide dilution; and 0.5 ml. of the enzyme dilution.

Enzyme preparation with spec. act. of 138,000 was diluted to 1.23×10^{-3} mg of prot. per ml.

Results:

	v of hydrolysis at (S) x 10^4M conc. of substrate				
pH	0.50	2.50	10.0	29.0	50.0
3.0	-	-	-	30	-
3.3	-	24	28	31	31
3.6	17	23	26	29	29
3.9	17	22	24	28	29
4.2	14	19	22	27	30
4.5	10	15	16	21	25
4.8	7.0	10	12	14	18
5.2	4.2	5.8	6.5	8.3	10
6.0	1.0	2.1	2.3	3.5	4.2

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