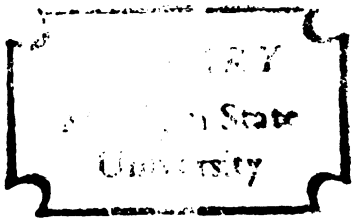
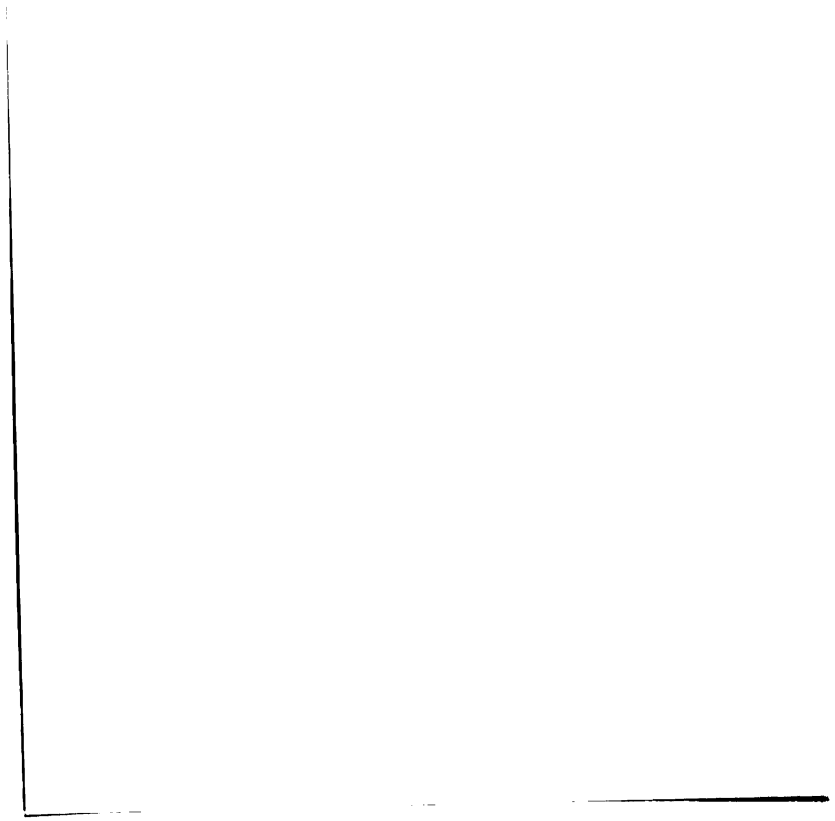
THE ISOLATION OF β-GLUCURONIDASE FROM FROM THE AQUATIC SNAIL, AMPULLARIA CUPINA

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
Patricia Josephine Casey
1960



MICHIGAN STATE UNIVERSITY



THE ISOLATION OF β-GLUCURONIDASE FROM THE AQUATIC SNAIL, *AMPULLARIA CUPINA*

By

Patricia Josephine Casey

A THESIS

Submitted to the College of Science and Arts of
Michigan State University in partial
fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Chemistry

1960

ACKNOWLEDGMENT

The author wishes to express appreciation and gratitude to Doctor John C. Speck, Jr. for his guidance and counsel.

Gratitude is also expressed to the National Institutes of Health for the financial support of this study.

To Jack

TABLE OF CONTENTS

Page

I. Historical Introduction 1

II. Experimental 5

1. Apparatus 5

2. Reagents 6

3. Assay of β-glucuronidase activity 9

4. Biuret protein determination 9

5. Column chromatography 10

6. Procedure for the isolation of β-glucuronidase from *Ampullaria cupina* 10

III. Results and Discussion 15

Bibliography 21

LIST OF TABLES

Table — Page

I Purity and Activity of β-Glucuronidase at different Stages of Purification 17

LIST OF FIGURES

Figure

1. Chromatography on a DEAE Cellulose Column 18

2. Chromatography on an IRC-50 Column 19

3. Effect of Temperature on β-Glucuronidase Activity 20

I. HISTORICAL INTRODUCTION

β-glucuronidase has been found in various plant and animal tissues. Although this enzyme has been known for some time and is widely distributed among plants and animals, the exact role it plays in metabolism is yet to be discovered. It has been a useful tool for the hydrolysis of glucuronides, especially in the study of steroid conjugates. Much has been done to link β-glucuronidase levels with specific pathological conditions. Due to the lack of purity of the enzyme preparations, little has been found about the specificity and mode of action of this enzyme. It therefore seems important to locate a source from which a pure preparation can be obtained.

Röhmann (1) and Serra (2) both observed glucuronidase activity in animal tissue extracts. Masamune (5) however, first described an extract of animal tissue containing an enzyme specific for the hydrolysis of β-glucuronides, and gave it the name β-glucuronosidase. Oshima (6, 7) found this enzyme in dog tissue and developed a better method of extraction.

Fishman (8) in 1938 began investigating the enzyme because of its possible utility in the hydrolysis of urinary conjugated steroids. The preparation he obtained was purified about 140-fold. Fishman's procedure involved extraction of ox spleen with water, followed by acetone precipitation and water extraction to pH 5.0, reduced in volume by evaporation, and fractionated with ammonium sulfate. The method of assay used at that time involved oxidizing the liberated glucuronic acid with ceric sulfate. Fishman continued his investigation of β-glucuronidase activity.

He observed the effect of feeding various substrates on the production of the enzyme by the animal body (10).

Graham (11) used 1-menthol glucuronide as substrate and defined the activity unit as the amount of enzyme which would liberate 0.100 milligrams of glucuronic acid. Graham was able to obtain a higher purity by the following procedure: the ox spleen tissue was minced in acetone and the precipitate extracted with tap water. The pH was adjusted to 5.0 and a series of ammonium sulfate precipitations carried out, finally yielding a preparation of 315-fold purity.

In 1946 Talalay, Fishman, and Huggins (12) developed an assay for glucuronidase activity in which the phenolphthlein released by the enzymatic hydrolysis of phenolphthlein mono-β-glucuronide was measured colorimetrically. Since this required only colorimetric determination of a hydrolysis product, it was a great improvement over the previous methods. The colorimetric measurement of the freed phenolphthalein was superior to the colorimetric measurement of estriol liberated from estriol glucuronide, because the phenolphthalein compound was so much easier to obtain. The phenolphthalein mono-β-glucuronide was biosynthesized by feeding phenolphthalein to rabbits and isolating the glucuronide from the urine of the animals.

It is this assay that was used to define the most commonly used unit of glucuronidase activity, the Fishman unit. One Fishman unit is defined as that quantity of enzyme which liberates one microgram of phenolphthalein in one hour at 37°C. from phenolphthalein glucuronide at pH 4.5. The use of the term Fishman unit has, however, been extended

by many to mean the quantity of enzyme which liberates one microgram of phenolphthalein under conditions of assay specified by the investigator. The usual variable in these assays is pH, since enzyme preparations from different sources have different pH optima.

Further attempts to purify β-glucuronidases from many different sources have been made and products of greater purity and activity have been obtained (13, 14, 15, 16). Data on the richness of individual tissues of different organisms has been complied (16). In addition to the wide distribution of this enzyme in mammalian tissues and secretions, it has also been found in fish liver (18), the crop fluid of locusts (16), and several species of mollusks (20, 21, 22, 23). A number of strains of microorganisms have been found to be good sources of this enzyme (16).

Despite purification of β-glucuronidase preparations in earlier work it was not until 1958 that Alfsen and Jayle (21) obtained a crystalline enzyme. This crystalline β-glucuronidase was obtained from the gastric juice of the land snail, Helix pomatia, and was found to be homogeneous on electrophoresis and ultracentrifugation. The procedure for obtaining the crystals consisted of ammonium sulfate fractionation, absorption of impurities on a tricalcium phosphate gel, and finally precipitation with ethanol. The crystals appeared on dialysis, after the final ethanol precipitation. The gastric juice contained the enzyme in a fairly concentrated solution, and was obtained from the duct between the stomach and hepatopancrease of the animal. The crystals were found to have a purity of 1.2×10^5 Fishman units per milligram of protein.

It was the isolation of crystalline β-D-glucuronidase from Helix pomatia that suggested the present investigation of Ampullaria cupina. Ampullaria cupina is a species of aquatic snail that inhabits tropical waters. It is a rather large snail and is available commercially. One also may obtain Ampullaria cupina from various streams in Florida.

II. EXPERIMENTAL

1. Apparatus

Spectrophotometers. The Beckman model B spectrophotometer and matched 6-inch soft glass test tubes were used for measurements in the visible range. The Beckman DU model spectrophotometer and 1-cm. quartz cells were used for measurements of absorbance in the ultraviolet region.

Centrifuges. The International Clinical Centrifuge running at three-quarter's full speed was used throughout, with the exception of the centrifugation of fraction VI which was carried out in the International model HR-1 centrifuge at 18,000 r.p.m. (40,000 x gravity).

Tissue Homogenizer. A Potter Elvehjem homogenizer was used.

Dialysis Apparatus. A beaker placed on a slowly rotating disk in which the dialysis bag was suspended was used. The apparatus was kept in the cold room at 4°C. A beaker supported in a circulating water bath at 0°C with a reservoir of saturated ammonium sulfate connected above the bath at room temperature was used when a slow increase in concentration desired. Visking cellophane tubing was used for all dialysis.

pH Meter. A Beckman model H2, glass electrode, line operated pH meter was used.

Chromatography Columns and Fraction Collector. A pyrex column 22 cm. long having an inside diameter of 1 cm. and a 200 ml. reservoir was used. The tip was connected to the tapered end by means of Tygon tubing and the flow was controlled by means of a screw clamp. A Misco fraction collector and timer were used.

Constant Temperature Baths. A Warburg apparatus water bath maintained at 37-38°C for the incubation of the enzyme preparations for assay. A Forma circulating water bath maintained at 0°C was used for low temperature dialysis.

2. Reagents

Buffers. Phosphate buffer 0.075 M, pH 6.8 (used in the enzyme activity assay of fractions I through IX).

10.2 g. potassium dihydrogen phosphate monohydrate

700 ml. water

Adjust to pH 6.8 with 10 per cent sodium hydroxide and dilute to 1 l. with water.

Glycine buffer 0.2 M, pH 10.4 (used to develop color in the assay of enzyme activity).

15.0 g. glycine

11.7 g. sodium chloride

700 ml. water

Adjust to pH 10.4 with 10 per cent sodium hydroxide and dilute to 1 l. with water.

Acetate buffer 0.1 M, pH 4.5 (used in the enzyme activity assay).

8.2 g. sodium acetate

7.73 ml. glacial acetic acid

700 ml. water

Dilute to 1 l. with water.

Phosphate buffer 0.10 M, pH 7.0 (used to elute the IRC-50 column).

7.16 g. sodium dihydrogen phosphate

28.8 g. disodium hydrogen phosphate

Dilute to 2 l. with water.

Phosphate buffer 0.005 M, pH 7.0 (used to elute DEAE columns).

Dilute 0.10 M, pH 7.0 phosphate buffer 1:20.

Phosphate buffer 0.01 M, pH 7.0 (used to elute DEAE columns).

Dilute 0.10 M, pH 7.0 phosphate buffer 1:10.

Phosphate buffer 0.02 M, pH 7.0 (used to elute DEAE columns).

Dilute 0.1 M, pH 7.0 phosphate buffer 1:5.

Phenolphthalein Standard Solution. (used as standard for the assay of enzyme activity).

Dilute 1 ml. of a 1 mg. per ml. phenolphthalein in 95 per cent ethanol solution to 50 ml. with 0.2 M, pH 10.4 glycine buffer.

Phenolphthalein mono-β-Glucuronide Solution 0.0015 M. (used as substrate in the assay for enzyme activity).

70 mg. phenolphthalein mono-β-glucuronide (Sigma)

Dilute to 100 ml. with water.

Biuret Reagent. (used for protein content determinations).

1.5 g. cupric sulfate pentahydrate

6.0 g. sodium potassium tartrate tetrahydrate

Dissolve in water and add 300 ml. of freshly prepared carbonate-free 10 per cent sodium hydroxide with stirring. Dilute to 1 l. and store in a polyethylene bottle.

IRC-50, XE-64 Fine Ground Resin. (used for column chromatography).

Suspend resin in buffer and discard those particles which do not settle out immediately.

DEAE-SF Resin. Exchange capacity 0.46 milliequivalents per gram. Control number CXD 602. (used for column chromatography).

Used as supplied.

Acetone. (CP grade) was used for tissue homogenization and for fractionation of the enzyme preparations.

Tricalcium Phosphate Gel. (used in an attempt to remove impurities from the enzyme preparation) (21).

150 ml. of calcium chloride dihydrate (132 g./ml.) diluted to 1600 ml.

150 ml. of sodium phosphate dodecahydrate (152 g./ml.)

Adjust to pH 7.4 with acetic acid. Wash precipitate six times with water a total volume of about 24 l. The precipitate collected by filtration in a Buchner funnel (without suction) overnight and stored in a glass container in the dark.

Bovine Serum Albumin Standard Solution.(used as standard for the biuret protein determination).

100 mg. bovine serum albumi, crystallized (Pentex)

Dissolve inwater and allow to stand overnight in refrigerator so that foam will settle, this solution then diluted to 100 ml..

All reagents used were of reagent grade unless otherwise specified.

3. Assay of Glucuronidase Activity

An aliquot of the enzyme preparation was pipetted into a test tube and the volume adjusted to 0.5 ml. with water. To this was added 0.5 ml. of buffer (either pH 6.8 phosphate buffer or pH 4.5 acetate buffer, depending on the pH desired) and 0.5 ml. of 0.0015 M phenolphthalein mono-β-glucuronide and the tubes immediately placed in a water bath maintained at a temperature of 37-38°C. The reaction was allowed to proceed at this temperature for 30 minutes and then was stopped by the addition of 5.0 ml. of 0.2 M pH 10.4 glycine buffer. The absorbance of the solution was measured at 540 mu.

4. Biuret Protein Determination (25, 26, 27)

An aliquot of the enzyme preparation was pipetted into a test tube and the volume brought to 5.0 ml. with water. Five ml. of the biuret reagent was then added with mixing and the tubes allowed to stand at room temperature for 30 minutes or longer. The absorbance was then measured at 540 mu.

5. Column Chromatography

Chromatography on IRC-50 columns. (28) The resin was equilibrated with 0.1 M pH 7.0 phosphate buffer and introduced into the column as a slurry. Particles which settled slowly were removed with a pipet before addition of more of the slurry. This was repeated until the resin reached a height of 15 cm.. Two ml. of the enzyme preparation then was allowed to percolate into the resin. The column was then eluted with 0.1 M pH 7.0 phosphate buffer. Eight fractions of approximately 1.5 ml. were collected.

Chromatography on DEAE cellulose columns. (29) The resin was equilibrated with 0.005 M pH 7.0 phosphate buffer and introduced into the column as a slurry until a height of 15 cm. was reached. The buffer was run through the column until the eluate reached a pH of 7. Two ml. of the enzyme solution was allowed to percolate into the resin. The column was then eluted in a stepwise fashion with 50 ml. each of the following buffers: 0.005 M, 0.01 M, 0.02 M phosphate pH 7.0. The fractions collected from the first column were approximately 5.5 ml. in volume and from the second column 5.0 ml. The resin was washed with 0.1 M pH 7.0 phosphate buffer and water before it was extruded from the column and repacked as described above.

6. Procedures for the Isolation of the β-Glucuronidase from Ampullaria cupina

A. The shells were removed from the snails with the aid of scissors, needle clamp and clippers. The foot was removed along with part of the

reproductive tract and respiratory system. The remaining tissue which consisted primarily of the hepatopancreas, but including the intestine and crop was cut into small pieces and homogenized with water in a potter Elvehjem homogenizer. The homogenate was centrifuged in a clinical centrifuge for 10 minutes. The precipitate (fraction I) was discarded.

The supernatant (fraction II) was adjusted to 35 per cent saturation by the addition of solid ammonium sulfate and centrifuged for 10 minutes in the clinical centrifuge. The precipitate (fraction III) was discarded. The supernatant (fraction IV) was adjusted to 70 per cent saturation by adding solid ammonium sulfate and centrifuged in the clinical centrifuge for 20 minutes. The supernatant (fraction V) was discarded.

The precipitate (fraction VI) was taken up in water and centrifuged in the International Centrifuge at 18,000 r.p.m. (40,000 x gravity) for 30 minutes at -2°C. The number 856 head was used. The precipitate (fraction VII) was discarded. The supernatant (fraction VIII) was dialyzed for 42 hours against water at 4°C. After dialysis, the ammonium sulfate concentration was adjusted to 25 per cent saturation by adding solid ammonium sulfate. No precipitate formed. The ammonium sulfate concentration was then increased with solid ammonium sulfate to 70 per cent saturation. The solution was allowed to stand overnight in the refrigerator and then centrifuged in the clinical centrifuge for 30 minutes. The supernatant (fraction XI) was discarded.

The precipitate (fraction X) was taken up in water and dialyzed at 4°C for 48 hours against glass-distilled water. After dialysis an equal weight of acetone was added and the solution centrifuged in the

clinical centrifuge for 20 minutes. The precipitate was discarded. The supernatant (fraction XII) was dialyzed against glass-distilled water for 45 hours at 4°C. An acetate buffer pH 4.5 was substituted for the phosphate pH 6.8 buffer used in the assay for enzyme activity up to this point.

After dialysis the ammonium sulfate concentration was adjusted to 70 per cent saturation with solid ammonium sulfate and the solution centrifuged. The supernatant (fraction XIV) was discarded. The precipitate (fraction XIII) was suspended in a small volume of water.

Aliquots of fraction XIII were tested for activity after having been heated to temperatures of from 45° to 65°C for 5 minutes. The remainder of fraction XIII was brought to a volume of 10 ml. with 0.1 M, pH 7.0 phosphate buffer. Five ml. of tricalcium phosphate gel was added and the pH adjusted to 5 and the mixture stirred for 5 minutes. The gel was then centrifuged for 15 minutes in the clinical centrifuge. The supernatant (fraction XV) was discarded. The precipitated gel and adhering protein (fraction XVI) was mixed for 10 minutes with 10 ml. of 0.1 M, pH 7.0 phosphate buffer and then centrifuged for 15 minutes in the clinical centrifuge. The supernatant contained no activity and no further attempts to elute the enzyme were made.

All fractions were assayed for β-glucuronidase activity and protein content before continuing with the fractionation.

B. The shells were removed and the tissue cut as described above. The tissue was then homogenized in cold acetone and centrifuged for 10 minutes in the clinical centrifuge. The precipitate was washed

twice with acetone and desiccated over phosphorous pentoxide overnight. The supernatant and washings were discarded.

The precipitate was suspended in water and adjusted to 20 per cent saturation with solid ammonium sulfate, and then centrifuged for 20 minutes. The precipitate (fraction 3) was discarded. The supernatant (fraction 2) was adjusted to 70 per cent saturation with solid ammonium sulfate and centrifuged for 15 minutes. The supernatant (fraction 5) was discarded.

The precipitate was taken up in water and dialyzed against 20 per cent saturated ammonium sulfate for 124 hours at 4°C. After dialysis, the solution was centrifuged for 10 minutes and the precipitate (fraction 6) was discarded. The supernatant (fraction 7) was heated to 60-65°C for 10 minutes and then centrifuged for 15 minutes. The precipitate (fraction 8) was discarded and the supernatant (fraction 9) adjusted to 40 per cent saturation by adding solid ammonium sulfate and allowed to stand overnight at 0°. The suspension was centrifuged. The supernatant (fraction 11) was discarded and the precipitate (fraction 10) was dialyzed against 0.1 M, pH 7.0 phosphate buffer for 48 hours at 4°C.

A 2 ml. aliquot of fraction 10 was put on the IRC-50 column as described above. Eight fractions of approximately 1.5 ml. were collected (fractions 10 IRC-50 1-8).

The remainder of fraction 10 was dialyzed against 0.005 M, pH 7.0 phosphate buffer at 4°C for 48 hours. A 2 ml. aliquot was put on a DEAE column as described above. Fractions of approximately 5.5 ml. were collected (fractions 10 DEAE 1-27). At this point, due to the

low concentration of the protein, protein content was determined by the absorbance at 280 mu.

Fraction 10 DEAE 21 was adjusted to 20 per cent saturation by the addition of solid ammonium sulfate and then dialyzed against an ammonium sulfate solution, the concentration of which was increased from 20 to 40 per cent saturation by adding saturated $(NH_4)_2SO_4$ solution over a period of approximately 2 days. The dialysis was allowed to continue for approximately 2 days longer and then solid ammonium sulfate was added slowly to increase the saturation to 50 per cent. Before all the ammonium sulfate crystals were dissolved a portion of the solution in the dialysis bag was removed and examined under the microscope at a magnification of 440. Well formed crystals were found to be present.

Another 2 ml. aliquot of fraction 10 was put on a DEAE column as described above and 30 fractions of approximately 5.0 ml. were collected, (fraction 10 DEAE II 1-30). Fraction 10 DEAE II 22 was pervaporated to a volume of about 2 ml. and then dialyzed against a solution of ammonium sulfate the concentration of which was increased from 20 to 50 per cent saturation over a period of 6 1/2 hours by the addition of saturated ammonium sulfate solution. After continuing the dialysis overnight no crystals were found in the aliquot removed. After 2 days crystals were seen at a magnification of 440.

III. RESULTS AND DISCUSSION

The enzyme β-glucuronidase has been isolated in a purified form from the hepatopancreas and digestive tract of Ampullaria cupina. The acetone powder was extracted with water and fractionated with ammonium sulfate. After a heat precipitation the preparation was chromatographed on a DEAE cellulose column. This procedure yielded crystals which were not assayed due to the small amount of crystals present. The purity of the solution, in one experiment, before crystallization was 71,428 Fishman Units per milligram of protein. This was a 168-fold purification from the first fraction assayed in this series. (Table I).

The use of the DEAE cellulose column seemed to be the most useful step in the fractionation procedure. The column was eluted in a stepwise manner with pH 7 phosphate buffers of increasing molarity; 0.005 M, 0.01 M, 0.02 M. The β-glucuronidase was recovered in a sharp peak just after the solvent was changed to 0.02 M buffer. (Fig. 1).

Several of the fractionation steps employed did not increase the purity of the enzyme enough to make them practical. Chromatography on the IRC-50 resin increased the purity about two-fold. The column was eluted with 0.1 M, pH 7.0 phosphate buffer. The enzyme was eluted verr rapidly and recovered in a clear peak (Fig. 2).

Heat precipitation gave very little purification and part of the enzyme was lost in this step. The inactivation of the enzyme when subjected to various temperatures is shown in Fig. 3.

An attempt to absorb impurities with a tricalcium phosphate gel was unsuccessful since the enzyme was not recovered. This gel might,

however, be useful if the enzyme is selectively absorbed and a method of elution developed.

The tissue from five large snails was used in each of the two series of fractionations. The weight of the whole snail including shell was from 10 to 20 g. The amount of the purified product obtained was very small. However, from the results obtained it seems that *Ampullaria cupina* is a probable source for crystalline β-glucuronidase.

TABLE I

Purity and Activity of β-Glucuronidase at Different Stages of Purification

Fraction	FU*/ml.	FU/mg protein
2	2,480	425
4	2,960	871
7	2,600	590
9	3,000	882
10	3,840	1,138
10 IRC-50	1,680	2,800
10 DEAE 21	360	24,000
10 DEAE II 21	200	71,428

*FU = Fishman Unit.

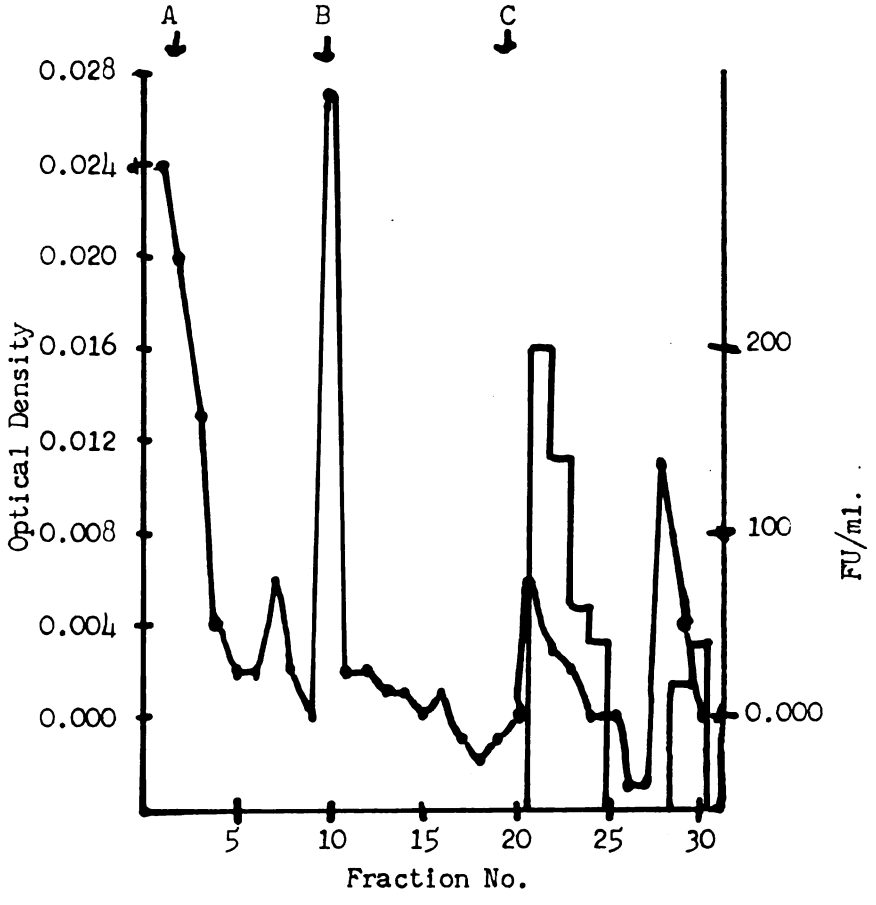


Figure 1. Chromatography on a DEAE column. A 2 ml. aliquot offraction 10 was chromatographed on a 1 x 15 cm column of DEAE cellulose. The column was equilibrated with 0.005 M, pH 7.0 phosphate buffer and then eluted with 50 ml. each of pH 7.0 0.005 M (A), 0.01 M (B), and 0.02 M (C) phosphate buffers. Fractions of 5.0 ml. volume were collected and the absorbance at 280 mu and β-glucuronidase activity measured. Block graph shows β-glucuronidase activity. Line graph shows optical density.

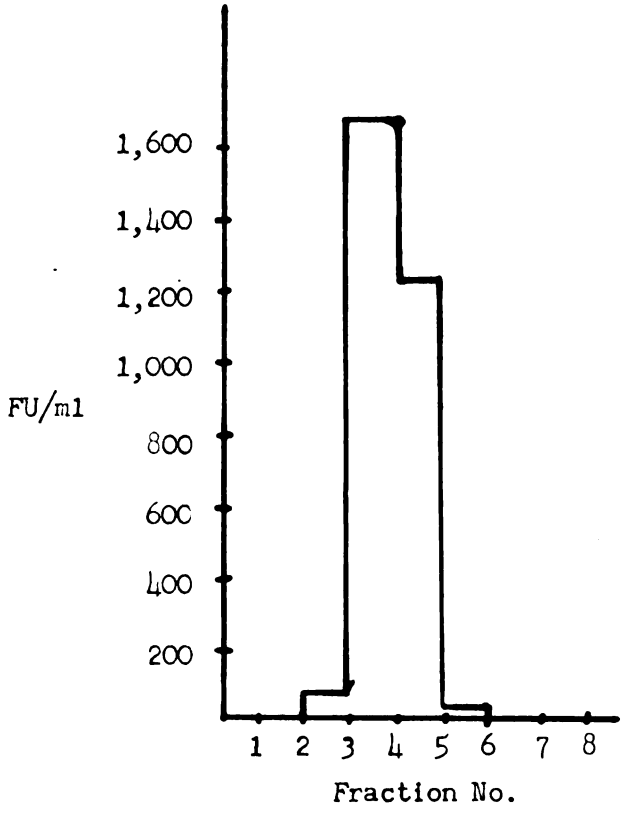


Figure 2. Chromatography on an IRC-50 column. A 2 ml. aliquot of fraction 10 chromatographed on a 1 x 15 cm column of IRC-50. The column was equilibrated with 0.1 M, pH 7.0 phosphate buffer and eluted with the same buffer. Fractions of 1.5 ml. volume were assayed for β-glucuronidase activity.

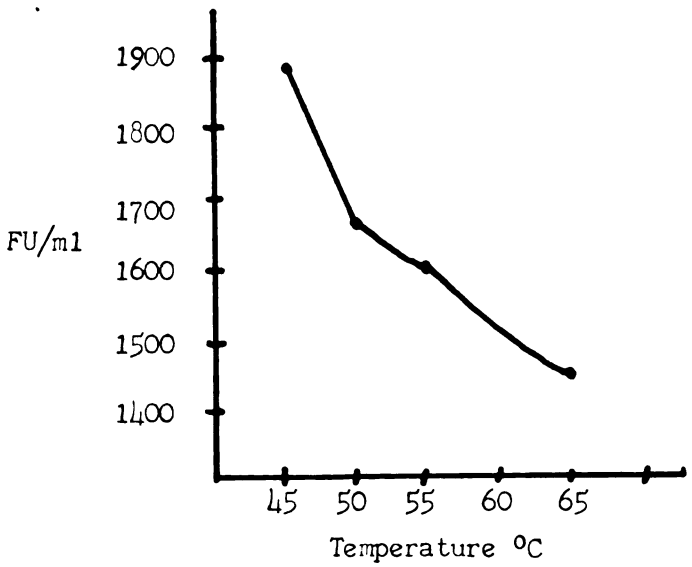


Figure 3. Effect of temperature on β-glucuronidase activity. Separate aliquots were heated to the temperature indicated for 5 minutes and then assayed for β-glucuronidase activity.

BIBLIOGRAPHY

(1) Rohmann, F. Biochemie (1908), quoted in H. Masamune, J. Biochem, (Japan) 19, 353 (1934).

(2) Serra, Y. Z. physiol. Chem. 92, 261 (1914).

(3) Serra, Y. ibid. 90, 258 (1914).

(4) Serra, Y. ibid. 88, 460 (1913).

(5) Masamune, H. J. Biochem. (Japan) 19, 353, (1934).

(6) Oshima, G. ibid. 20, 361 (1934).

(7) Oshima, G. ibid. 23, 305 (1936).

(8) Fishman, W. H. J. Biol. Chem. 127, 367 (1939).

(9) Fishman, W. H. ibid. 131, 225 (1939).

(10) Fishman, W. H. ibid. 136, 229 (1940).

(11) Graham, A. F. Biochem. J. 40, 603 (1946).

(12) Talalay, P., W. H. Fishman, and C. Huggins. J. Biol. Chem. 166, 757 (1946).

(13) Smith, E. E. B., and G. T. Mills, Biochem. J. 53, 164 (1953).

(14) Bernfield, P., J. S. Nisselbaum, and W. H. Fishman. J. Biol. Chem. 202, 763 (1953).

(15) Levvy, G. A., A. McAllen, and C. A. Marsh. Biochem. J. 69, 22 (1958).

(16) Fishman, W. H. Advances in Enzymol. 16, 361 (1955).

(17) Fishman, W. H., in "The Enzymes", Ed. J. B. Summer and K. Myrback, Academic Press Inc. Publishers, New York, 1, 635 (1950).

(18) Neuberg, C. and A. Graver, Enzymologia 15, 115 (1951).

(19) Robinson, D., J. N. Smith, and R. T. Williams, Biochem. J. 53, 125 (1953).

(20) Jarrige, P., and R. Henry, Bull. soc. chim. biol. 34, 872 (1952).

(21) Alfsen, A., and M. F. Jayle, ibid. 40, 2143 (1958).

(22) Billet, F. Biochem. J. 57, 159 (1954).

(23) Stitch, S. R., I. D. K. Halkerston, and J. Hillman, ibid. 63, 705 (1956).

(24) _____. Bulletin 105, Sigma Chemical Company, St. Louis (1958).

(25) Carter, H. E. Experimental Biochemistry, Stipes Publishing Co., Champaign, Ill. 132, (1959).

(26) Gornall, A. G., C. J. Bardwall, and M. M. David. J. Biol. Chem. 177, 751 (1959).

(27) Robinson, H. W., and C. G. Hogdern, ibid. 135, 727 (1940).

(28) Moore, S., and W. H. Stein. Advances in Protein Chem. XI, 191 (1956).

(29) Ellis, O. Biochem. J. 233, 63 (1958).

(30) Dixon, M., and E. C. Webb. Enzymes, Academic Press Inc., Publishers, New York (1958).