THE ENZYMATIC HYDROLYSIS OF NITROGEN

DIOXIDE OXIDIZED CELLULOSE

by
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I   INTRODUCTION

In 1936, W. O. Kenyon, of the Eastman Kodak Co., discovered that cellulose could be oxidized with nitrogen dioxide to produce a new type of oxidized cellulose, in which oxidation occurred almost completely at carbon six, resulting in a polyuronic acid structure. The product of this selective oxidation was designated as _celluronic acid_, in order to indicate its polyuronic acid structure and to distinguish it from other types of oxidized celluloses, which are included under the generic name of oxycellulose.*

Several years later it was found that celluronic acid gauze could be used in surgery as an absorbable hemostatic sponge.[1,2,3] It proved to be outstanding for this purpose because, first, unlike other types of oxidized cellulose, this material retained the fibrous structure of cellulose and possessed a high enough tensile strength so that it would resist handling; second, the presence of carboxyl groups on the cellulose chain caused the material to be soluble in dilute alkali and indeed at blood pH (7.4); and third, it was found to produce no anaphylactic reaction on implantation in body cavities.

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*It must be remembered that the term celluronic acid, as used for nitrogen dioxide-oxidized cellulose, does not represent a chemically homogeneous species, as will be evident from the discussion of its structure (see below). The term nitrogen-dioxide oxidized cellulose would be preferable from a structural point of view but is more cumbersome to use.

The success of celluronic acid gauze as an absorbable surgical sponge with hemostatic properties led to large-scale production[4] and marketing by pharmaceutical companies for medical use.

Although, as stated above, celluronic acid is soluble at blood pH, the details of its absorption from body cavities and assimilation or elimination are not well understood. Microscopic examination of sponge packs several days after implantation shows the presence of large number of macrophages (large white cells) surrounding the packs[5,6] indicating their involvement in the disintegration process, while the tissue juices may also have some action.[1]

It occurred to us that the process of absorption of celluronic acid in the body might involve the enzymatic hydrolysis of the material to glucuronic acid or lower-molecular weight poly-glucuronic acids. We therefore undertook the study of the action of enzymes on celluronic acid and an examination of the products of this action.

II   THE PREPARATION AND STRUCTURE OF CELLURONIC ACID

Celluronic acid is commercially available in the form of a powder or gauze. It is manufactured by the vapor phase oxidation of cotton cellulose (powdered cellulose or surgical gauze), followed by washing with water and drying in warm air.

A detailed study of the oxidation reaction[7] has shown that it involves preliminary nitration of the cellulose, presumably at carbon six, followed by oxidative denitration at this position. The product retains a small amount (less than 0.5%) of combined nitrogen, which may be in the form of nitrate groups on carbons two or three.

Celluronic acid has been shown by various analytical methods to be chiefly a copolymer of glucuronic acid and glucose. The following is a brief review of the results on which this structure is based.

Like other uronic acid polymers or uronic acids themselves, celluronic acid evolves carbon dioxide on refluxing with dilute hydro-chloric acid.[8] The decarboxylation may be made quantitative, and is commonly used for the analysis of polyuronides. On application of this method to celluronic acid, the amount of carbon dioxide produced was found to depend on the extent of oxidation of the cellulose, up to the maximum theoretical value of 25.0%, which corresponds to a polyglucuronic acid, i.e., complete oxidation of the carbon six positions in cellulose. Kinetic studies also showed that the rate curve of carbon dioxide evolution from celluronic acid was similar to that obtained with alginic and pectic acids.[9]

This method of analysis was found to be the most reliable, and manufactured celluronic acid is therefore characterized by its carbon dioxide equivalent. The commercial product contains carboxyl groups corresponding to 18-20% carbon dioxide, hence 72-80% of the glucose units have been oxidized.

A second proof of structure is the formation of furfural on heating with hydrochloric acid, under the conditions used for the determination of pentoses by this reaction.[10] The yield of furfural is only 60% of the amount expected in comparison with pentosans.

Further proof of structure has been provided by progressive oxidation according to the following scheme:[11]
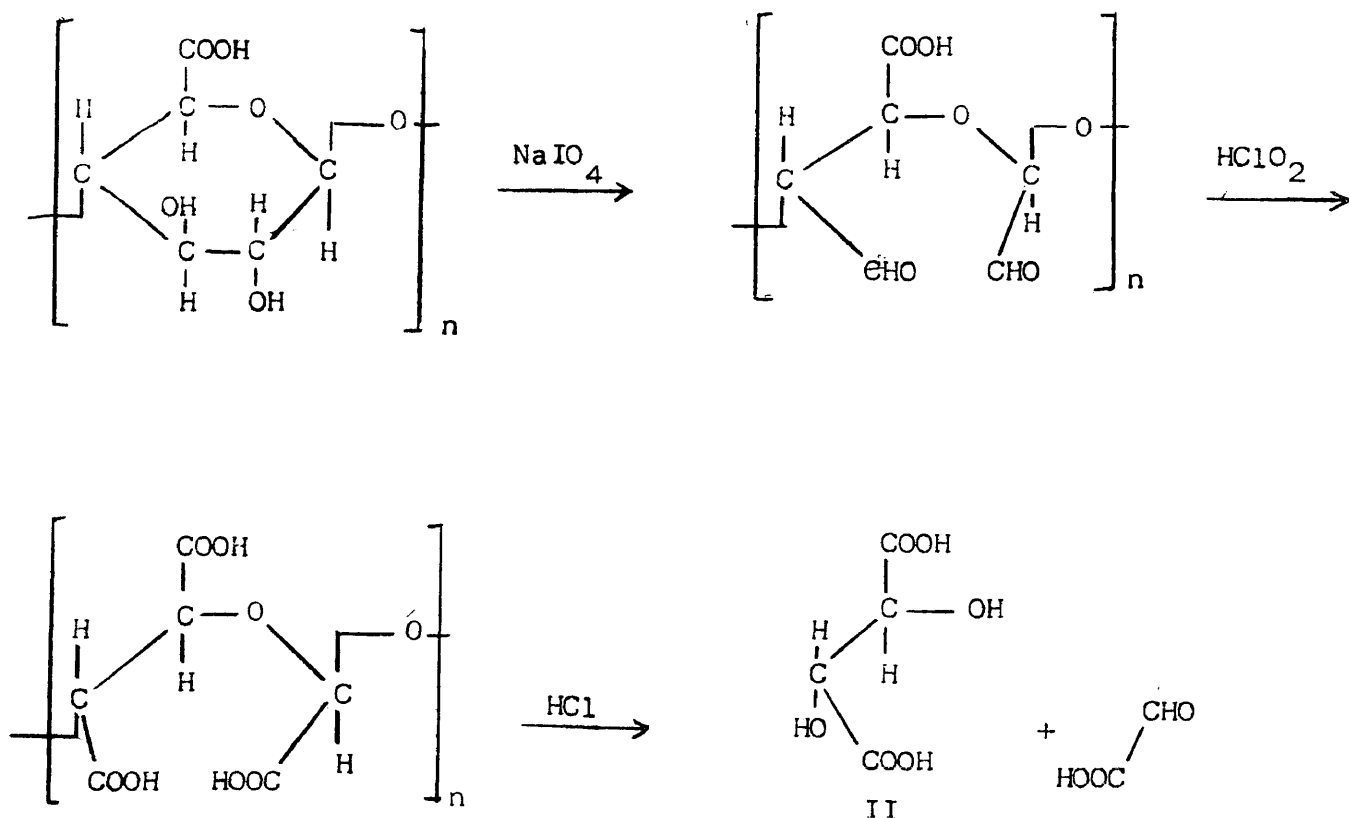
Mesotartaric acid (II) was produced in an overall yield of 28% of theory. A control experiment with alginic acid gave a 27% yield of the same product. This experiment indicates the above structure (I) for celluronic acid, although carbon six might be either an aldehyde or a carboxyl group.

Celluronic acid shows the acidic properties to be expected from its structure, forming water-soluble sodium, ammonium, quaternary ammonium and pyridine salts, and a water-insoluble barium salt. It has a strong affinity for basic dyes.[8] Direct titration of celluronic acid with sodium hydroxide gives a neutralization equivalent which is too low; i.e., base is consumed beyond the amount required to neutralize a uronic acid. Experiments have shown that exposure of the celluronic acid to a pH above 7 causes this phenomenon, with which is associated a profound degradation of the celluronic acid. An alternative means of titration of celluronic acid was found by first treating the material with either calcium acetate[8] or sodium bromide,[12] liberating acetic or hydrobromic acid respectively, which may then be titrated with alkali. Reproducible results were obtained, but the carboxyl content calculated in this manner was always lower than that obtained by the carbon dioxide evolution method.

This discrepancy has been interpreted by the investigators as being due to lactone or anhydride formation during the oxidation of the cellulose, so that some of the carboxyl groups are not available for exchange with cations below a pH which would cause saponification. A similar discrepancy was found in the case of pectic substances, between the carboxyl content as determined by the calcium acetate method and that given by carbon dioxide evolution.[13]
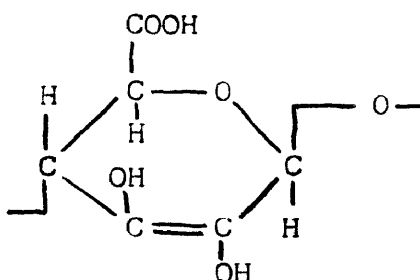
The sensitivity of celluronic acid to alkalies led the investigators

to the conclusion that there was an additional structural feature

of the material which had not been considered. It was found by

reaction with O-methylhydroxylamine, under slightly acidic conditions,

that celluronic acid contains small amounts of carbonyl groups (up

to two per cent of the weight of celluronic acid).[14] Since the

physical properties of the celluronic acid indicate that little de-

gradation of the polymer chain has occurred in the oxidation process,

and since the conditions for reaction with O-methylhydroxylamine

also would not cause hydrolysis with concomitant liberation of

aldehyde groups, the logical conclusion was drawn that the celluronic

acid contains ketonic or aldehydic groups within the glucuronic

acid or glucose residues in the chain. The sensitivity of aldehydes,

as compared with ketones, to nitrogen dioxide, indicates that the

former would not survive the oxidation process. Thus by elimination,

the source of the reaction with O-methylhydroxylamine was ascribed

to ketonic groups at carbons two or three of the pyranose ring.

The infrared spectrum of celluronic acid[15] shows absorption

peaks at 6.1 μ and 7.8 μ which have been ascribed to the carboxyl

groups, as well as the 2.9 μ peak due to hydroxyl. A fourth peak

at 5.7 μ falls in the carbonyl region and has been considered

evidence for the presence of keto groups by Kenyon, et al.[14] However,

this value is actually closer to the value expected for anyhydrides

than to that expected for ketones.

The mechanism by which ring ketonic groups may cause alkali

sensitivity is evident on consideration of the work of Evans and

co-workers on the alkaline hydrolysis of glycosides,[16] which was

applied to certain types of oxidized celluloses by Ivanov and Kaversneva.[17]

Evans' results showed that a glycosidic link becomes labile
to alkali if a double bond is attached to the glycosidic carbon,
or if one may arise in that position by enolization. Thus, di-
hydroxyacetone glucoside was found to be hydrolyzed by alkali.

As was pointed out by Kenyon,[14] this principle applied to
the celluronic acid case predicts that the presence of a carbonyl
group on carbons two or three of the glucose or glucuronic acid
residue in the chain would render the adjacent glucosidic link
sensitive to alkali; in alkaline solution the following structure
will arise, which will be a weak point in the chain.

The sensitivity of celluronic acid to alkalies has the im-
portant consequence that the common methods of analysis for
reducing groups cannot be applied for the determination of
end groups in this material. The copper number, for example, has
no meaning, since impossibly high values are always obtained.[14,18]

Other methods which are used for the determination of reducing
groups, such as the cerimetric method (see below) are carried out
under alkaline conditions and hence are not suitable for this
purpose. However, since celluronic acid appears to be fairly
stable to acid hydrolysis, reducing end groups could be determined
under mildly acid conditions. The O-methylhydroxylamine method dis-
cussed above measures end groups along with ketonic groups under
these conditions.

The results of Kenyon and co-workers on the preparation and properties of celluronic acid were confirmed by Maurer[19] who also studied the chemoimmunological properties of this substance,[20] as did Heidelberger.[21] Solutions of the sodium salt of celluronic acid behave immunologically like the capsular polysaccharide of Type III pneumococcus. This polysaccharide has been shown to consist of alternate glucopyranose and glucuronic acid residues.[22]

Maurer also prepared a blood anticoagulant by esterifying the hydroxyl groups of celluronic acid with chlorosulfonic acid.[23] This material was not as effective as heparin but was less toxic than cellulose sulfate preparations, due perhaps to the fact that the celluronic acid, which would be liberated by hydrolysis of the ester in the body, is soluble, in contrast to cellulose.

III PRELIMINARY OBSERVATIONS

In preliminary experiments, we found that a suspension of dried spleen in water caused the gradual disintegration and solution of celluronic acid gauze. On incubation at 37°, about two weeks were required for the disappearance of the gauze. In a control experiment in which the spleen was omitted, no change was observed in the appearance of the gauze over a period of several months, except for a slight swelling.

One of the enzymes which is present in spleen is β-glucuronidase, and it seemed possible that this enzyme might be responsible for the action of the spleen on celluronic acid. This hypothesis was strengthened by the fact that celluronic acid contains β-glucuronic acid linkages (i.e., those corresponding to the original β-glucosidic linkages of the cellulose). Accordingly, a commercial β-glucuronidase made from bacteria was next tried and found to have the same action on celluronic acid gauze as that of spleen. In contrast to this, a commercial polygalacturonase (pectinol) had no action on the gauze.

β-glucuronidase is very widespread in occurrence, both in plants and animals; in animals, the spleen is the richest source, but other tissues, including the blood, urine and white blood cells, have been demonstrated to contain the enzyme.[24] Since β-glucuronidase has not been obtained in crystalline form, it is not known whether preparations of this enzyme consist of only one enzyme or several. It has a pH optimum of 4-6 depending on the source of the enzyme and the substrate.

β-glucuronidase action is usually measured either by determina-
tion of the amount of aglycon liberated (as in the case of phenolphthalein
glucuronide) or by determination of the glucuronic acid set free. The
latter may be determined conveniently by the Tollens naphthoresorcinol
reaction[25] or by cerimetry.[26,27]

Neither of these methods can be applied to the β-glucuronidase-
celluronic acid system. Since the Tollens naphthoresorcinol test
depends on the development of color when glucuronic acid is heated
with naphthoresorcinol in hydrochloric acid solution, any polyuronic
acid will give a positive test due to hydrolysis of the glucosidic
linkages.

The cerimetric method involves heating the sample in a sodium
carbonate-potassium ferricyanide solution, with oxidation of aldehyde
groups and reduction of the ferricyanide to ferrocyanide, which is
then titrated with ceric sulfate. Our experiments showed that
celluronic acid itself gives a high reducing value by this method,
apparently for the same reason that it gives a high copper number.

The sensitivity of celluronic acid to both acid and alkali thus
rules out the common methods of determining reducing groups and
following the course of enzymatic hydrolysis by chemical means. The
use of O-methylhydroxylamine[28] or hydroxylamine[29] for the estimation
of reducing groups would not be suitable for kinetic work due to
experimental difficulties such as the time involved in carrying out
the analyses (eighteen hours).

In view of these difficulties, the only suitable criterion
which was found for enzyme action on celluronic acid gauze was the
actual disappearance of the gauze. However, it was also found that
the course of enzymatic action on a _solution_ of celluronic acid

(i.e., its sodium salt) could be followed by viscosimetry. Solutions

of celluronic acid at pH 6 were found to undergo some decrease in

viscosity on standing, but the addition of β-glucuronidase caused

a rapid decrease in viscosity, the rate of change of viscosity

appearing to be roughly proportional to the amount of enzyme present,

although sufficient data were not obtained to allow any quantitative

relationships to be developed. It was felt that any kinetic analysis

of the viscosimetric data was unwarranted, in view of the complexity

of the system, and the only use to which these measurements were

put was to indicate the practical completion of the hydrolytic

reaction.

IV   EXPERIMENTAL

A. Materials

Celluronic acid was obtained from the Tennessee Eastman Co. as "Oxycel" surgical gauze. The material was stated by the manufacturer to contain 20% carbon dioxide, i.e., 80% of the carbon six positions were oxidized to carboxyl.

The gauze was estimated to contain 12-13% adsorbed moisture. The exact amount must vary with atmospheric humidity. The moisture content was determined by drying a weighed sample of "air-dry" gauze over phosphorous pentoxide in a drying pistol at about 20 mm. pressure and room temperature. The change in weight with time is shown in Table 1 and the results shown graphically in Figure 1. When the weight had become practically constant, the pistol was heated with refluxing water and the gauze decreased further in weight, as shown in the upper curve of Figure 1. This latter change was accompanied by yellowing of the gauze, indicating decomposition, and suggesting that the water which was being removed at 100° was chemically bound.

Bacterial β-glucuronidase was obtained from the Sigma Chemical Co. Since the enzyme is not pure, it is assayed by the manufacturer. Three samples were used, analyzing 15,800, 19,700 and 25,000 units per gram, respectively.[*]

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[*]One unit of bacterial β-glucuronidase is defined as that amount of enzyme which will liberate one microgram of phenolphthalein from phenolphthalein glucuronide in one hour under specified conditions.

The dried spleen preparation used was Viobin 40° material;

Pectinol 10-M (Rohm & Haas) was used as a source of polygalacturonidase.

Glucurono-δ-lactone was obtained from the Corn Products Refining

Co. Glucuronic acid was prepared from the lactone by regeneration

from the barium salt, as described by Goebel and Babers.[30] All other

chemicals used were C.P. grade.

Table 1

Determination of Moisture Content of Celluronic Acid Gauze

Time of Drying	Weight of Sample	% Loss in Weight
0 hr.	0.4478 g.	0
24	.4038	9.8
49	.3962	11.5
71	.3938	12.0
139[a]	.3919	12.5
147	.3864	13.7
163	.3835	14.3
171	.3826	14.5

a
    After 139 hrs., sample was heated at 100°.

B. Viscosimetric data

Air-dried celluronic acid gauze (2.0 g.) was suspended in

water and dissolved by the dropwise addition of dilute sodium

hydroxide. The solution, which had a pH of approximately 6, was

then diluted to 50 ml. To one 10.0 ml. portion was added 0.20 g.

of bacterial β-glucuronidase (3200 units). The resulting solution

was placed in a modified Ostwald viscosimeter and set in a thermo-

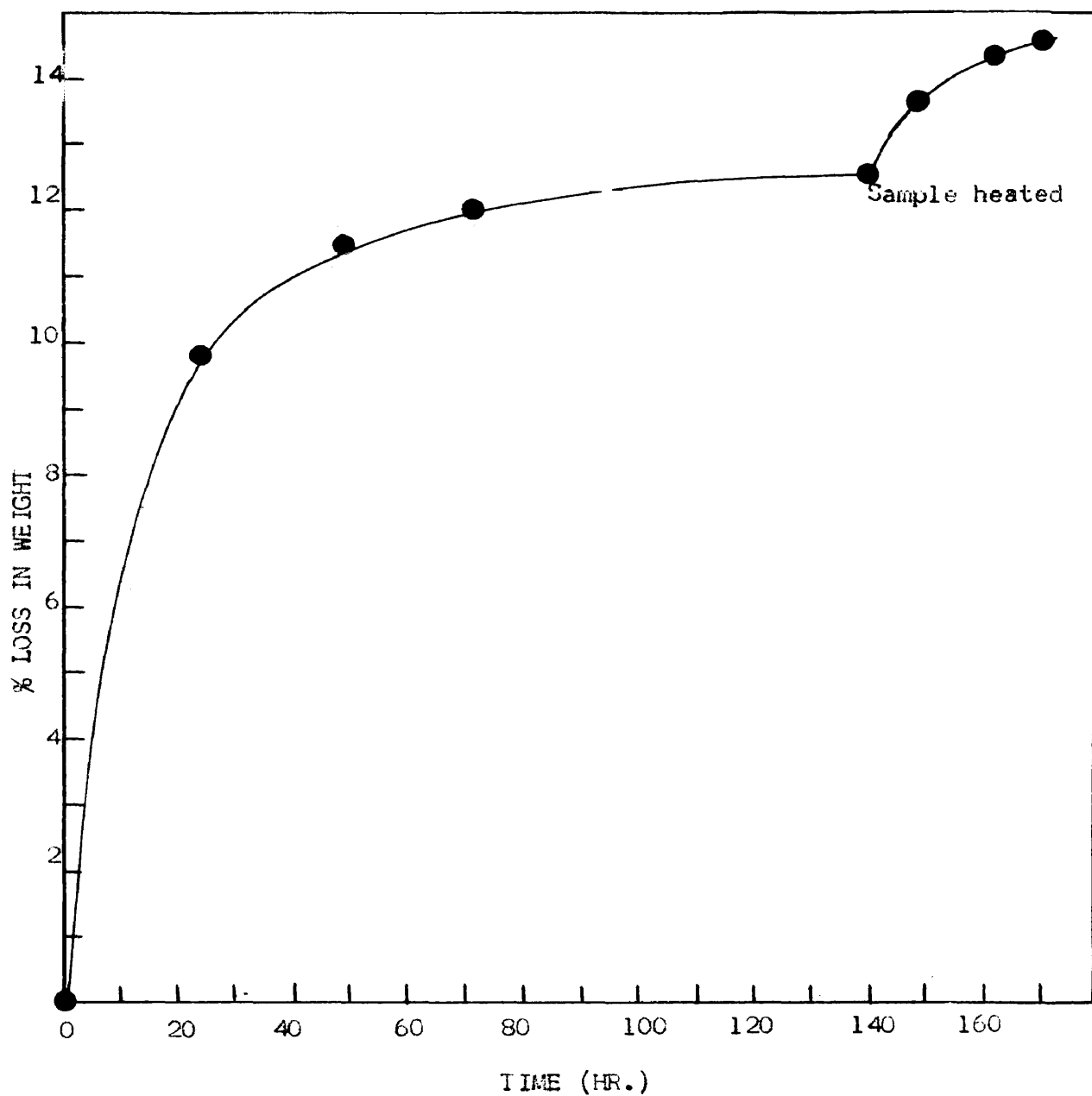statically controlled water bath at 37°±1°. A second 10.0 ml.

FIGURE 1

Determination of Moisture Content of Celluronic Acid

portion of the celluronic acid solution was placed in a duplicate viscosimeter in the bath as a control. A third viscosimeter contained a solution of 0.20 g. of bacterial β-glucuronidase (3200 units) in 10.0 ml. of water.

The viscosity of each of the three solutions was measured at intervals at the temperature of the bath, and corrected to 37.0° by means of an empirical factor derived from measurement of the change in viscosity of the solutions over the temperature range 36-38°. The precision of measurement of viscosity was ±1%. The viscosimeters were calibrated with distilled water.

The measured viscosities are given in Table 2. In Figure 2 are plotted the curves showing the decrease in viscosity with time, expressed as fraction of original viscosity. Curve C is that of the celluronic acid plus 0.20 g. of enzyme. Curve B is that of a second experiment in which 0.10 g. (1600 units) of the enzyme was used under conditions which were otherwise identical with those of the first experiment. Curve A was plotted for the control solution of celluronic acid without enzyme, using data from both experiments.

No change in viscosity was observed for the solution containing enzyme alone. The marked decrease in viscosity caused by the addition of the enzyme is apparent from Figure 2.

C. Preparation of enzyme hydrolyzate

A series of hydrolysis experiments was carried out to yield product for study of its structure. These experiments were of two types, those using celluronic acid gauze itself and those using a solution of celluronic acid at pH 6.

Table 2

Viscosimetric Data on Enzymatic Hydrolysis of Celluronic Acid

Exp. 1

		Viscosities (centistokes)	
Elapsed time (hr.)	Substrate	Substrate + enzyme[a]	Enzyme[a]
0	1.54	1.42	0.82
2.5	1.53	1.33	.82
20	1.47	1.14	.82
26	1.47	1.12	.81
46	1.41	1.07	.81
72	1.36	1.05	.82
117	1.31	1.03	.81

Exp. 2

		Viscosities (centistokes)
Elapsed time (hr.)	Substrate	Substrate + enzyme[b]
0	1.66	1.66
4	1.65	1.57
25.5	1.58	1.43
28	1.51	1.34
72	1.45	1.29
96	1.42	1.27
123	1.36	1.23
146	1.38	1.22

a
    Enzyme concentration 2% (3200 units/10 ml.)
b
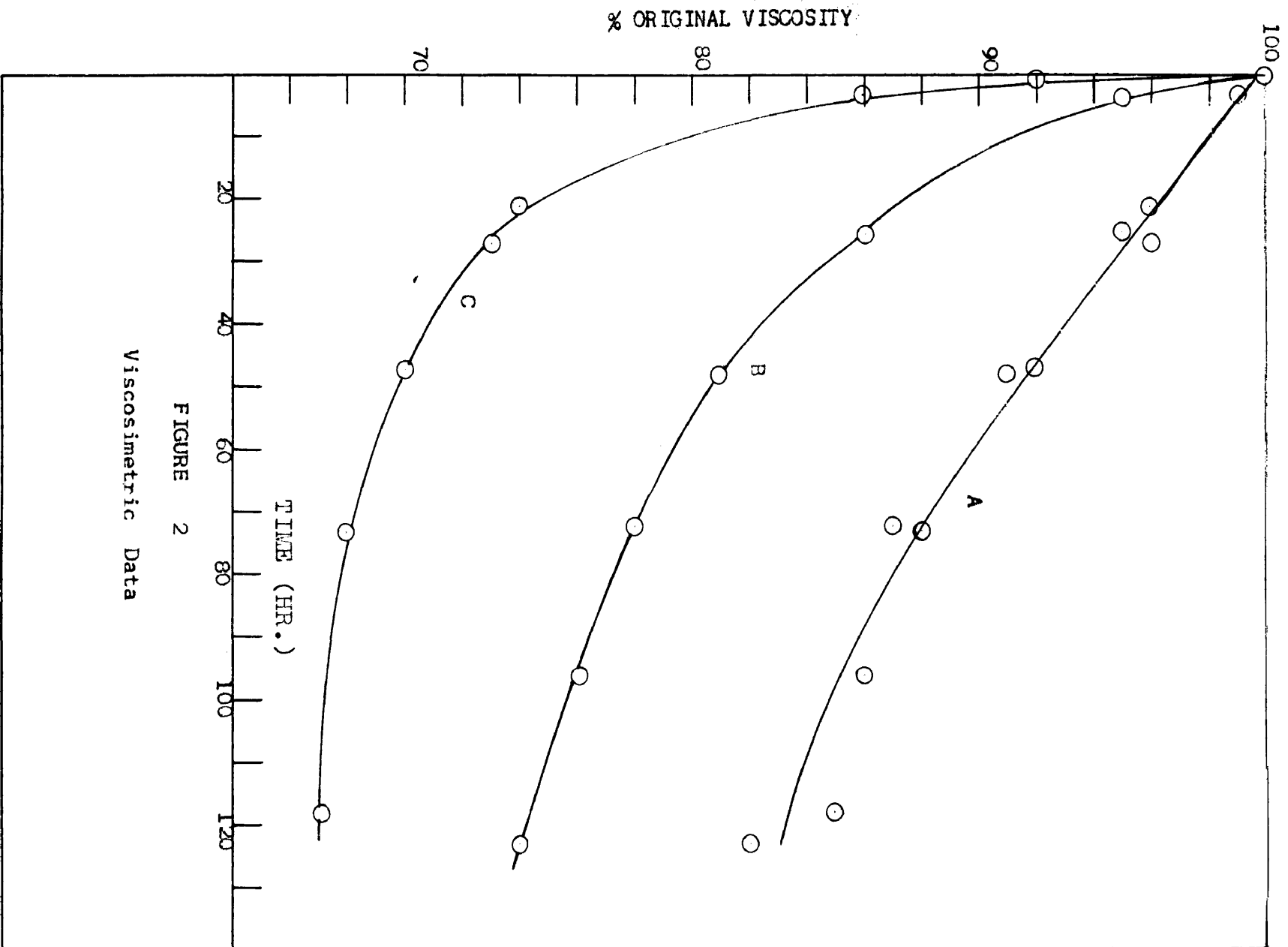   Enzyme concentration 1% (1600 units/10 ml.)

FIGURE 2

Viscosimetric Data

In the first type of experiment, the gauze was suspended in a solution of the enzyme and the end of the reaction estimated by the disappearance of the gauze. In the second type, the solution of celluronic acid was prepared as described under the discussion of viscosimetric experiments and the end of the reaction judged from the attainment of constant viscosity.

The time required for the hydrolytic reaction to proceed to completion was much less in the second type of experiment (about five days) than in the first type (two to four weeks).

Examples of the first type of experiment are given in Table 3, experiments 1-4; the second type is exemplified by experiments 5-9.

The products of hydrolysis were worked up in the same way for both types of experiments. At the end of the reaction, the solution was removed from the incubation bath and a few milliliters of a 20% aqueous solution of trichloroacetic acid was added. The solution was then concentrated to about half its volume at the water pump from a warm water bath (about 40°). This caused the precipitation of the enzyme as a gel, which was removed by centrifugation or filtration. The filtrate was further concentrated in the same manner, and additional enzyme removed if necessary. Finally a light brown clear sirup was obtained. On addition of an excess of absolute ethanol, the sirup solidified, giving an almost white amorphous solid, which was filtered, washed with absolute ethanol and ether, and dried.

D. Products of enzymatic hydrolysis

1. Preliminary examination of the enzyme hydrolyzate

The product obtained in this manner was a non-hygroscopic powder, freely soluble in water to give a clear solution. It was insoluble in all organic solvents. The aqueous solution was acidic, and addition

Table 3

Hydrolysis of Celluronic Acid with β-Glucuronidase[a]

Exp. No.	Wt. of substrate[b]	Wt. of enzyme	Units of enzyme	Total vol.	Incubation time	Yield of hydrolyzate	% Yield[c]
1	1.0 g.	0.70 g.	10,000	70 ml.	2 weeks	0.50 g.	57
2	4.0	1.0	15,800	70	4 "	1.70	48
3	2.0	1.0	15,800	70	3 "	1.04	57
4	2.0	1.0	15,800	70	3 "	0.8	50
5	2.0	1.0	15,800	60	5 days	0.50	28
6	2.0	1.6	31,600	60	5 "	0.57	32
7	2.0	1.6	31,600	60	4 "	0.60	34
8	2.0	1.0	19,700	75	5 "	0.52	29
9	2.0	0.6	16,00	60	5 "	0.84	48

[a]Exps. Nos. 1-4 with celluronic acid gauze; Nos. 5-9 with solutions os gauze at pH 6.

[b]Air-dry celluronic acid.

[c]Grams of product/g. of anhydrous celluronic acid.

of barium hydroxide precipitated a barium salt, from which the free

acid could be regenerated by treatment with sulfuric acid. It gave

a positive naphthoresorcinol test, and gave a precipitate with phenyl-

hydrazine or 2,4-dinitrophenylhydrazine.

The solubility of the hydrolyzate in water showed that its molecular

weight was obviously very low compared with either the starting material

or with alkali-degraded celluronic acid. The latter material was pre-

pared by dissolving celluronic acid gauze in dilute sodium hydroxide and

adding excess hydrochloric acid. This produced a translucent solution.

After recovery of the celluronic acid by evaporation of the solution it

was obtained as a white powder which could not be redissolved in water.

This behavior, which is characteristic of pectic or alginic acid, is

associated with a high molecular weight.

2. Partition chromatography experiments on the enzyme hydrolyzate

The products of enzymatic hydrolysis were subjected to filter paper

partition chromatography to determine whether they might be separated

into components and whether glucuronic acid might be detected therein.

An attempt was also made to hydrolyze the enzyme hydrolyzate further

by means of 80% sulfuric acid, which has been used for the analogous

hydrolysis of alginic acid to mannuronic acid.[31] For acid hydrolysis,

100 mg. of the enzyme hydrolyzate was dissolved in one milliliter of 80%

sulfuric acid. The solution was allowed to stand at room temperature for

four days, and was then diluted with water, and neutralized with an

excess of barium carbonate. The precipitated barium sulfate and the

excess barium carbonate were removed by filtration and the clear yellow

filtrate was concentrated to about one milliliter under reduced pressure.

Similar solutions of celluronic acid and of pectic acid were made
up by dissolving 3 g. of each in 10 ml. of 80% sulfuric acid. These
solutions were also allowed to stand at room temperature for four
days and then worked up as in the case of the enzyme hydrolyzate,
the solutions finally being concentrated to a few milliliters. This
caused the precipitation of a gelatinous material from the pectic
acid solution. All the solutions turned black during the sulfuric
acid hydrolysis, indicating considerable decomposition, and some gas
was liberated in each case, indicating decarboxylation.

Unidimensional chromatography of the hydrolyzed materials was
carried out in the usual type of apparatus by the descending technique[32]
using Whatman No. 1 filter paper strips. Various solvent mixtures were
tried. The one giving the best results was water-saturated isobutyric
acid.[33] The paper was spotted with droplets of the solutions under
investigation, and chromatography was carried out for 24 hours, the
solvent being allowed to drip off the end of the paper strip. The
strips were then removed, air dried, sprayed with ammoniacal silver
nitrate, and heated at 100° in order to develop the chromatograms.

The results of the chromatographic experiments are shown in
Figure 3. A control spot of glucuronic acid (A) moved 22 cm. down
the paper. The enzyme hydrolyzate (B) gave a streak extending 8 cm.
down the paper. The sulfuric acid treated enzyme hydrolyzate pro-
duced two spots, at 18.5 and 23.5 cm., while the sulfuric acid
treated celluronic acid gave only a streak covering most of the
chromatogram. Sulfuric acid treated pectic acid gave three spots,
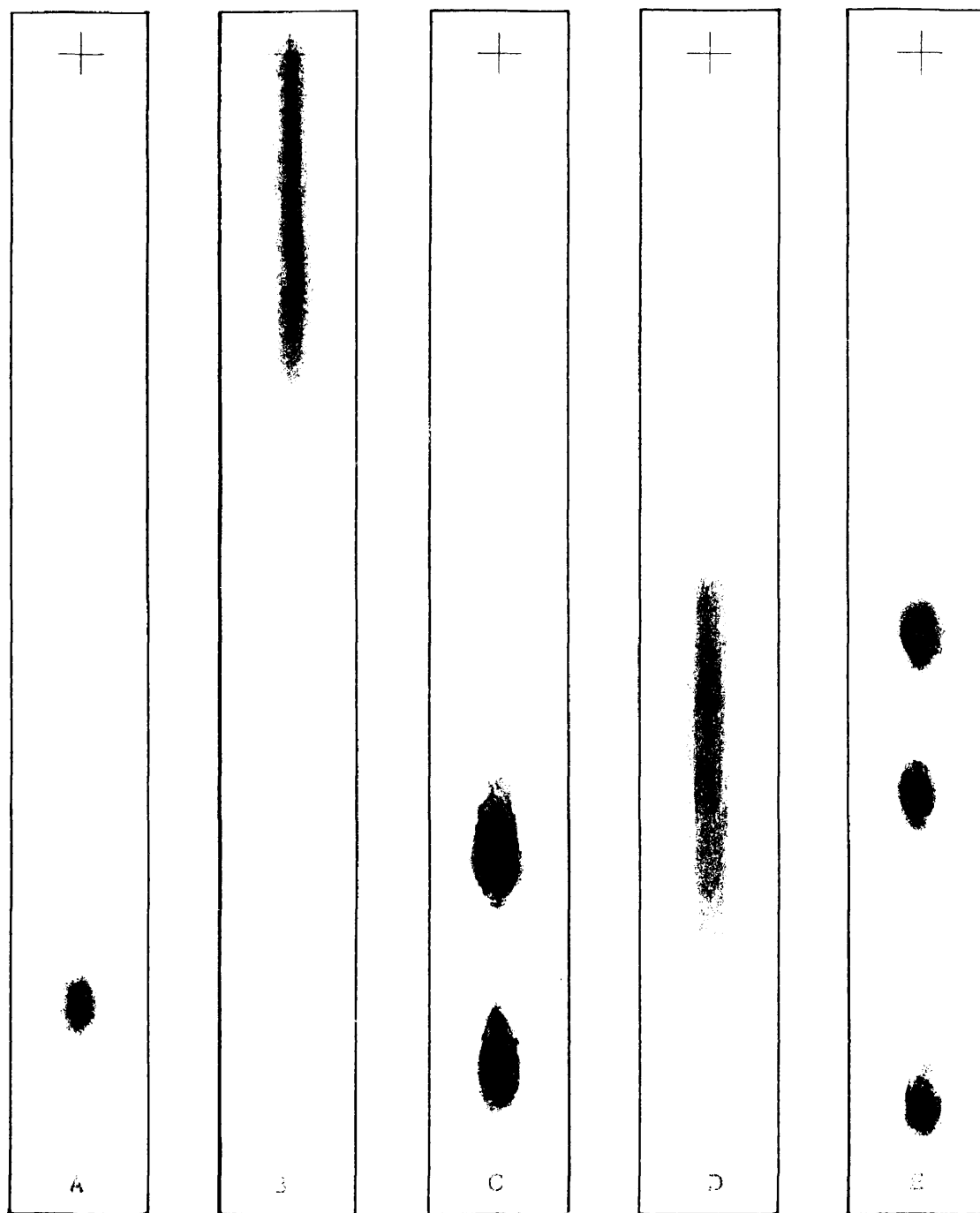at 13.5, 17 and 24.5 cm. from the starting position.

FIGURE 3
Partition Chromatograms

A-Glucuronic acid
B-Enzyme hydrolyzate
C-Enzyme hydrolyzate, acid hydrolyzed
D-Polluronic acid, acid hydrolyzed
E-Pectic acid, acid hydrolyzed

Other solvents which were tried, such as n-butanol-water, n-butanol-ethanol-water, and pyridine-ethyl acetate-water, gave less satisfactory results, since the enzyme hydrolyzate spot did not move down the paper.

3. Neutralization equivalent of the enzyme hydrolyzate

An aqueous solution of the enzyme hydrolyzate was poured through a column of cation-exchange resin (Amberlite IR-120) in order to convert any neutralized carboxyl groups to the free acid form, and the enzyme hydrolyzate was recovered by evaporation of the solution at reduced pressure. The neutralization equivalent of this purified hydrolyzate was then determined by potentiometric titration with 0.00950 N sodium hydroxide, using a glass electrode (Leeds and Northrup pH meter) to measure the endpoint. The sodium hydroxide was standardized against potassium hydrogen phthalate in the same manner. The titration curve for the enzyme hydrolyzate is shown in Figure 4, from which the neutralization equivalent for the enzyme hydrolyzate was estimated as 185.

4. Preparation of barium salt of the enzyme hydrolyzate

The hydrolyzate was dissolved in distilled water to give a dilute solution. Saturated barium hydroxide was then added drop-wise to precipitate the barium salt. Care was taken not to add excess barium hydroxide. Because of the appreciable solubility of the barium salt in water a small amount of 95% ethanol (about 20%) was added to the solution before the salt was filtered. The salt was then washed with dilute alcohol, and dried. From 1.39 g. of enzyme hydrolyzate were obtained 1.94 g. of barium salt (97-100% yield, depending on formula used; see Discussion below).
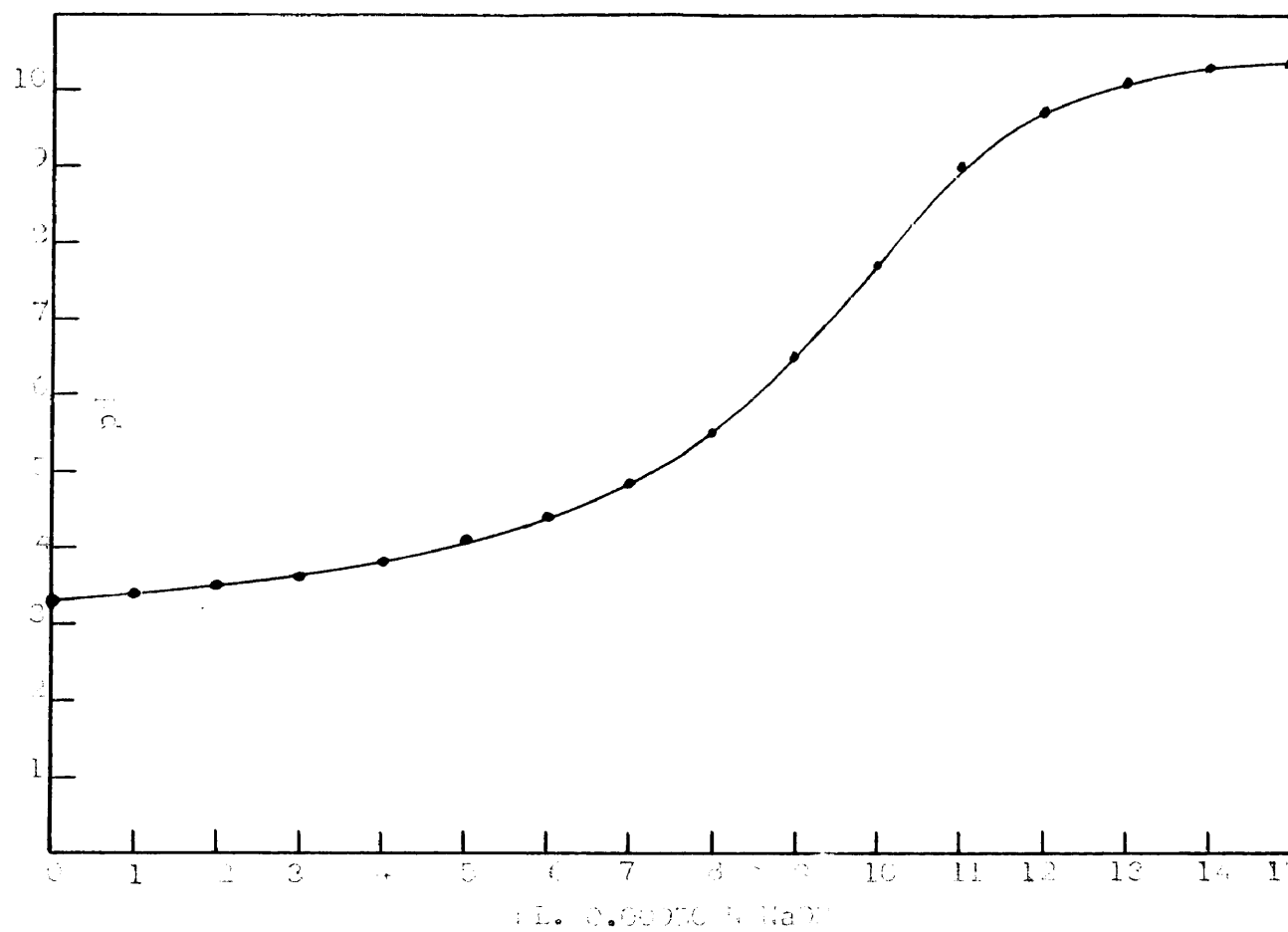
FIGURE 4

Titration Curve for Neutralization of Enzyme Hydrolyzate
Sample Weight 16.6 mg.

The barium salt was converted to the free acid by suspending it in distilled water and adding an equivalent amount of 1:1 sulfuric acid, followed by filtration of the barium sulfate and evaporation of the filtrate _in vacuo_ from a warm water bath. The free acid was precipitated by the addition of absolute ethanol. It was again converted to the barium salt, and the salt analyzed for barium content by quantitative precipitation of barium sulfate. Analysis: found, Ba 27.2 and 27.5%.

5. Preparation of sodium salt of the enzyme hydrolyzate

On complete neutralization with sodium hydroxide, the enzyme hydrolyzate gave a white crystalline sodium salt. This salt, which was very soluble in water but practically insoluble in organic solvents, was recrystallized from methanol-water.

Analysis: C 35.17%, H 3.82%, Na 11.64%

Optical rotation: $[\alpha]_D^{20}$ -21.6° (3.9%, 20.0 cm.)

6. Reaction of the enzyme hydrolyzate with 2,4-dinitrophenyl-
   hydrazine

The enzyme hydrolyzate (0.25 g.) was dissolved in a filtered solution of 0.25 g. of 2,4-dinitrophenylhydrazine in 40 ml. of 1.6 N hydrochloric acid. The solution was allowed to stand at room temperature. A partially crystalline precipitate was deposited which was filtered, washed with dilute hydrochloric acid and water, and dried. It weighed 0.30 g. It was purified by dissolving in methyl cellosolve and precipitating with ether. It melted at 195° with decomposition. Attempts at recrystallization led to decomposition.

Analysis: C 40.25%, H 4.85%, N 8.08, 7.86, 7.80, 8.05%*

The enzyme hydrolyzate also reacted with phenylhydrazine but gave an impure product which could not be worked up without decomposition.

The enzyme hydrolyzate sodium salt gave a crystalline 2,4-dinitrophenylhydrazine derivative under the same conditions which were used for the free acid. All attempts at recrystallization of the derivative led to decomposition. The major portion of the derivative was soluble in methanol and crystallized from this solvent by evaporation of the solvent at room temperature. The derivative melted at 192° with decomposition.

Analysis: N 8.54, 9.54%

Filter paper chromatography of these derivatives was carried out in an effort to determine whether they were homogeneous. The solvents used (ethanol-ethyl acetate-water 1:4:2 and ethanol-n-butanol-water 1:4:2) all gave single spots, which were however accompanied by considerable tailing. There was no evidence of separation into multiple spots.

7. Cerimetric analysis of the enzyme hydrolyzate

The analytical procedure used was that of Miller and Van Slyke[26] for the analysis of reducing sugars. It is based on the oxidation of the reducing sugar by heating with an excess of alkaline potassium ferricyanide solution, followed by titration of the ferrocyanide which is formed, with standard ceric sulfate solution.

The sample was heated for fifteen minutes in a boiling water bath with 20 ml. of a solution containing 5.00 g. of potassium

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*The analyst reported difficulties with the Dumas apparatus while the sample was being analyzed.

ferricyanide and 10.6 g. of anhydrous sodium carbonate per liter. The solution was then cooled in cold water and acidified by the addition of one milliliter of 65% sulfuric acid. A few drops of a 0.1% solution of Setopaline C indicator solution were added and the mixture was titrated with 0.00905 N ceric sulfate solution (standardized against standard ferrous ammonium sulfate solution).

The reducing equivalent weight of the enzymatic hydrolysis products was determined by using glucurono- δ -lactone as a reference standard. Analyzed by the above technique, 5.0 mg. of glucurono- δ -lactone required 17.2 ml. of 0.00905 N ceric sulfate (duplicate 17.2 ml.). Therefore one millimole of the lactone is equivalent to 612 ml. of 0.00905 N ceric sulfate. This value was used for the calculation of the equivalent weights given below, on the basis that one millequivalent of reducing power of the enzyme hydrolyzate would equal one millequivalent of glucurono- δ -lactone.

The reaction mixture resulting from enzymatic hydrolysis of celluronic acid gauze was analyzed by cerimetry to determine its reducing value. A 0.50 ml. aliquot of 10.0 ml. of the solution from the hydrolysis of 0.100 g. of celluronic acid gauze with 0.050 g. (750 units) of bacterial β -glucuronidase required 7.0 ml. of 0.00905 N ceric sulfate (duplicate 7.1 ml.). Calculated as above, the equivalent weight of the hydrolysis products is 440.

Blank determinations on the bacterial β -glucuronidase showed that, in the concentrations used, it had negligible reducing power.

The enzyme hydrolyzate, after recovery and purification via the barium salt, was analyzed in a similar manner. Duplicate samples,

each containing 9.7 mg. of enzyme hydrolyzate, required 11.8 and 12.0 ml. of ceric sulfate, respectively. This corresponds to an equivalent weight of 500. Further purification of the enzyme hydrolyzate via the barium salt did not change the equivalent weight.

In the same manner, the analysis of celluronic acid gauze gave the result that 5.0 mg. of air-dried celluronic acid corresponded to 6.1 ml. of ceric sulfate (duplicate 6.1 ml.). This corresponds to an equivalent weight of 480.

8. Acetylation of celluronic acid

For comparison with acetylation experiments on the enzyme hydrolyzate, celluronic acid itself was acetylated, under two different reaction conditions.

(a) Heterogeneous acetylation (without solution of the polymer): The method of Yackel and Kenyon[8] was used. Air-dried celluronic acid gauze (10.0 g.) was suspended in a mixture of 72 ml. of ligroin (65-90°) and 8 ml. of glacial acetic acid. The mixture was allowed to stand for 24 hours at room temperature. To the mixture were then added 30 ml. of glacial acetic acid, 45 ml. of acetic anhydride, 40 ml. of ligroin, 8 ml. of trichloroethylene, and 0.10 ml. of concentrated sulfuric acid. The resulting mixture was allowed to stand at 38° for four days. The fiber remained intact, although it swelled somewhat. At the end of this time, the gauze was removed and washed with ethanol, followed by water. It was dried in air. No appreciable change in weight of the air-dried material was observed on drying over alumina in vacuo. Yield 12.1 g.

For analysis, acetyl content was determined by saponification followed by acidification and steam distillation of the liberated acetic acid.[34] This method has been shown to be suitable for the analysis of acetylated celluronic acid.[8] Analysis: CH_3CO found, 32.0%. Yackel and Kenyon reported 33.0% acetyl for their product prepared in the same way.

(b) Homogeneous acetylation with a large amount of catalyst: Air-dried celluronic acid gauze (5.0 g.) was suspended in a mixture of 20 ml. of glacial acetic acid and 20 ml. of acetic anhydride. The suspension was warmed briefly in hot water and one ml. of concentrated sulfuric acid was added with shaking. The gauze quickly dissolved to give a clear yellow-brown solution which was then cooled and poured with stirring into about 500 ml. of water. The product was filtered, washed with water and dried. Yield 6.8 g.

Analysis: CH_3CO found, 37.6, 38.2%

The relative extent of degradation of these acetylation products may be estimated from the following considerations. The material prepared by procedure (a) retained its fibrous structure and was insoluble in chloroform or acetone, while that prepared by procedure (b) was soluble in acetone and partially soluble in chloroform. Evaporation of the solvent from this solution left the acetate as a colorless transparent film.

9. Acetylation of the enzyme hydrolyzate

The hydrolyzate was acetylated under two reaction conditions, corresponding to procedures (a) and (b) which were applied to celluronic acid.

(a) Heterogeneous acetylation (using an inert solvent): The enzyme hydrolyzate (0.10 g.) was suspended in a mixture of 7 ml. of ligroin (b.p. 60-90°) and one milliliter of glacial acetic acid. The reaction mixture was allowed to stand at room temperature overnight. Then 2 ml. of glacial acetic acid, 5 ml. of acetic anhydride, 4 ml. of ligroin, one milliliter of trichloroethylene and one milliliter of a solution of one milliliter of concentrated sulfuric acid in 10 ml. of glacial acetic acid were added. The reaction was allowed to proceed at 38° for 48 hours. At the end of this time the enzyme hydrolyzate had dissolved to give a dark colored clear solution. The solvent was removed by evaporation at reduced pressure and the residue triturated with water, filtered, and dried. It weighed 0.11 g. It was purified by dissolving in acetone, filtering, and evaporating the solvent. After drying over phosphorous pentoxide at reduced pressure, it was analyzed for acetyl content. Analysis: CH_3CO found, 34.6, 34.7%

(b) Homogeneous acetylation with a large amount of catalyst: The enzyme hydrolyzate (1.0 g.) was suspended in a mixture of 4 ml. of acetic anhydride and 4 ml. of glacial acetic acid and warmed briefly in hot water. Six drops of concentrated sulfuric acid were added with shaking, and the mixture was then allowed to stand for two hours at room temperature. At the end of this time the clear, colorless solution was poured onto 25 g. of ice and the white precipitate filtered. The precipitate was dried in air, dissolved in acetone, and precipitated as an amorphous powder by pouring into excess ether. It weighed 0.44 g.
Analysis: C 45.36%, H 5.11%; CH_3CO found, 35.0, 35.4%

10. **Reaction of the enzyme hydrolyzate with diazomethane**

The enzyme hydrolyzate (0.50 g.) was dissolved in a few milli-
liters of water, cooled in an ice bath, and shaken with portions of
a cold ethereal solution of diazomethane (prepared from nitrosome-
thylurea[35]) until the ether layer retained a yellow color. The
excess diazomethane was removed by warming the mixture, and the
ether decanted. The aqueous solution was then filtered to remove
a trace of insoluble matter, and evaporated to dryness in vacuo.
Trituration with ether and filtration yielded a yellow amorphous
powder, weight 0.47 g. In contrast to the acidity of aqueous
solutions of the enzyme hydrolyzate, the solution of the product
of diazomethane treatment was neutral, indicating complete esteri-
fication had occurred.

After the material had been purified by several-fold pre-
cipitation with ethanol from aqueous solution and evaporation with
water at reduced pressure to remove ethanol, the molecular weight
was determined by freezing-point depression in water. A solution
of 263 mg. in 15.0 ml. of water caused a freezing-point depression
of 0.075°. This corresponds to a molecular weight of 440 $\pm$ 20.

11. **Molecular weight of the enzyme hydrolyzate by freezing-
point depression**

An effort was made to determine the molecular weight of the
enzyme hydrolyzate from the freezing-point depression of aqueous
solutions. A sample of enzyme hydrolyzate was purified by passage
of its aqueous solution through a column of cation-exchange resin
(Amberlite IR-120) for the removal of cations. A solution of 100 mg.

of this material in 15.0 ml. of water showed a freezing point depression of 0.050°, corresponding to a molecular weight of 250 ± 10.

This value appeared to be too low in comparison with other analytical values for the molecular weight. A possible source of error was located in the work of Zechmeister and Toth[36] on the similar problem of determining the molecular weight of oligosaccharides produced in the hydrolysis of cellulose. These workers found that the apparent molecular weight of the oligosaccharides, as determined by freezing point depression in water, varied greatly depending on the history of the sample. The common method for purification of materials of this nature is solution in water followed by precipitation with a water-miscible non-solvent such as ethanol. Apparently the product after this treatment retains considerable adsorbed alcohol, which is not removed by drying in vacuo. In order to remove this alcohol, it was necessary to evaporate the sample to dryness from water solution several times, finally drying over phosphorous pentoxide. As an example, the apparent molecular weight of a tetrasaccharide purified by precipitation with ethanol and dried over phosphorous pentoxide in vacuo was 202. After removal of ethanol by the above treatment, the apparent molecular weight rose to 588.

A sample of enzyme hydrolyzate was therefore purified in the usual manner via the barium salt, followed by precipitation with ethanol from aqueous solution, and the ethanol then removed by repeated evaporation with water at reduced pressure. A solution

of 246 mg. of this material in 15.0 ml. of water gave a freezing

point depression of 0.038°. This corresponds to a molecular weight

of 800 $\pm$ 40.

12. <u>Regeneration of the enzyme hydrolyzate from its sodium salt</u>

The crystalline sodium salt of the enzyme hydrolyzate (0.32 g.)

was dissolved in water and passed through a column of Amberlite IR-120

cation exchange resin in order to regenerate the free acid. Evapora-

tion of the effluent gave an amorphous white solid (0.25 g.). The

neutralization equivalent was found by potentiometric titration to

be 230. Repetition of the ion exchange treatment gave a material

with a neutralization equivalent of 234.

The reducing value which was found for this material by cerimetry

was essentially the same as that of the enzyme hydrolyzate. Duplicate

samples, of 10.0 mg. each, required 13.7 and 13.8 ml. of 0.00905 N

ceric sulfate, respectively, corresponding to an equivalent weight

of 470.

Reaction of a sample of this product with 2,4-dinitrophenyl-

hydrazine under the conditions used in section 6 gave a red

crystalline substance, which was recrystallized from methanol at

room temperature. It melted at 192° with decomposition.

Analysis: C 42.83%, H 4.27%, N 12.61%

13. <u>Action of bacterial β-glucuronidase on cellobiose</u>

A solution of 40 mg. of cellobiose and 40 mg. (1000 units) of

bacterial β-glucuronidase in 4.0 ml. of water (pH adjusted to 6

with acetic acid) was incubated at 37° and the reducing power of

a 0.20 ml. aliquot determined at intervals by the cerimetric method.

The results are shown in Table 4, from which it may be seen that there was no appreciable change in the reducing power of the solution, indicating that the bacterial β-glucuronidase did not hydrolyze the cellobiose.

Table 4

Elapsed time	Ml. 0.00905 N ceric sulfate
0 hr.	7.0
6	7.2
24	7.4
48	7.3
72	7.3

V   DISCUSSION

From the results which have been presented it may be seen that
the action of bacterial β-glucuronidase on celluronic acid is the
production of a water-soluble product, the properties of which
correspond to those of a low-molecular-weight oligo-glucuronic acid.

Unfortunately, no completely reliable index of the molecular
weight of the product has been found. The progressive decrease in
the viscosity of solutions of celluronic acid plus enzyme points to
a decrease in chain length of the substrate. The much slower decrease
in viscosity of the substrate in the absence of enzyme may be due
to chemical hydrolysis of the celluronic acid chain, although this
point has not been investigated.

The absence of any appreciable amount of glucuronic acid in
the hydrolysis product is shown by two facts. First, the reducing
equivalent weight of the total hydrolysis product (440) is only
slightly below that of the enzyme hydrolyzate which was recovered
and purified (500). Any appreciable amount of glucuronic acid in
the crude hydrolyzate solution would have caused the equivalent
weight to be much lower.

Second, the experiments on paper chromatography of the puri-
fied enzyme hydrolyzate showed that this material contained no
monomeric glucuronic acid.

The data obtained from cerimetric analysis of the enzyme
hydrolyzate are of limited value, and are not a true index of
the actual molecular weight of the hydrolyzate. This is seen from

the fact that cellurcnic acid itself has approximately the same

equivalent weight by cerimetry (480) as does the enzyme hydrolyzate.

The equivalent weights which would be calculated for a poly-

glucuronic acid on the basis of one reducing group per molecule are

shown in the following table:
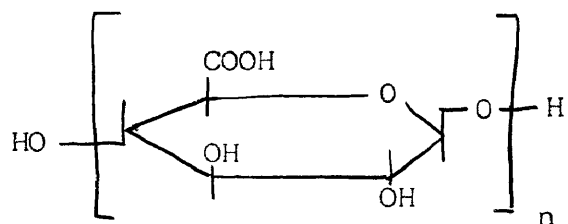
n	Molecular or equivalent weight
1	194
2	370
3	546
4	722

Thus the only conclusion that may be drawn from the cerimetric

analysis of the enzyme hydrolyzate (equivalent weight found 500)

is that its average molecular weight must be 500 or above.

The neutralization equivalent which was found for the enzyme

hydrolyzate was 185. For a polyuronic acid with the above structure,

the neutralization equivalents corresponding to different chain

lengths are shown in the following table.

n	N.E.
1	194
2	185
3	182
4	181
5	180
large	176

Thus, while the neutralization equivalent which was found corresponds to a value of $n = 2$, it cannot be used to decide the value of n with any accuracy.

The analysis of the barium salt which was obtained showed it to contain 27.4% barium. The barium content of a salt with the following structure varies with chain length as shown in the accompanying table.
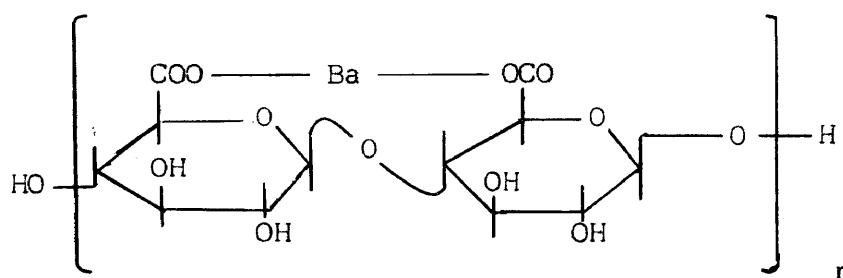
n	% Ba
1	27.2
2	27.6
3	27.8
4	27.9
5	27.9
large	28.1

The barium analysis fits a formula with $n = 1.5$, i.e., containing three glucuronic acid residues. However, again the analysis cannot decide the exact molecular weight.

The analytical data for the crystalline sodium salt (35.17% C, 3.82% H, 11.64% Na) are also in agreement with those calculated for the sodium salt of a polyglucuronic acid of the following formula, but again do not differentiate clearly between different chain lengths.
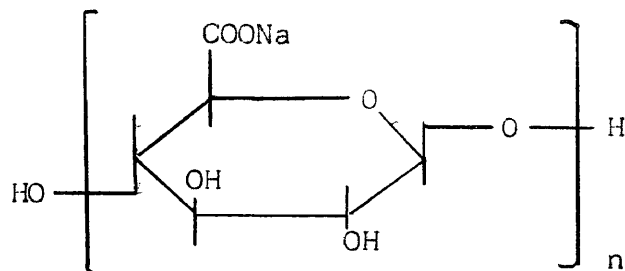
n	%C	%H	%O	%Na
2	34.8	3.9	50.3	11.1
3	35.3	3.8	49.7	11.3
4	35.6	3.7	49.4	11.4

The acetylation experiments also indicate the correctness of the polyuronic acid formula, without giving any clue to the molecular weight. Under very mild reaction conditions, viz., at 38° with a small amount of catalyst in the presence of a large excess of inert solvent, celluronic acid itself is acetylated to give a product, containing 32.0% acetyl, which is insoluble in the reaction mixture or in acetone. Under identical reaction conditions, the enzyme hydrolyzate gives a product soluble in the reaction mixture, and in acetone, and which contains 34.7% acetyl.

Under somewhat more rigorous reaction conditions (larger amount of catalyst and no inert solvent) celluronic acid gives an acetone-soluble product containing 37.9% acetyl. Under these conditions, the enzyme hydrolyzate gives apparently the same product as that obtained above, with 35.2% acetyl.

For celluronic acid itself, on the basis of a starting material in which 80% of the glucose units have been oxidized (i.e., a 20% carbon dioxide product) the acetyl content of a completely acetylated material would be 35.7%, if no degradation occurred. Any degradation

would be accompanied by an increase in the acetyl content of the product. Therefore the acetyl content of the acetylated celluronic acid (37.9%) prepared by procedure (b), together with the fact that it is soluble in acetone whereas product (a) is not, indicates that degradation has occurred in procedure (b).

On the other hand, the fact that the acetyl contents and solubility behavior of acetylated enzyme hydrolyzates prepared by procedures (a) and (b) are the same, indicates that procedure (b) caused no more degradation than did procedure (a). Since the conditions of procedure (a) are known to cause very little degradation,[37] it may be assumed that little degradation of the enzyme hydrolyzate occurred in procedure (b), also.

It should be noted that the procedure which has been used to give a cellulose acetate less degraded than that given by any other method, namely, heating with acetic anhydride and pyridine,[38] could not be used in the present case. Although it has been used successfully for the acetylation of pectic acid,[39] the application of this method to celluronic acid caused extensive decomposition of the celluronic acid, with the formation of a brown tar.

The calculated values for partially and totally acetylated polyglucuronic acids with different chain lengths are given in the following table.
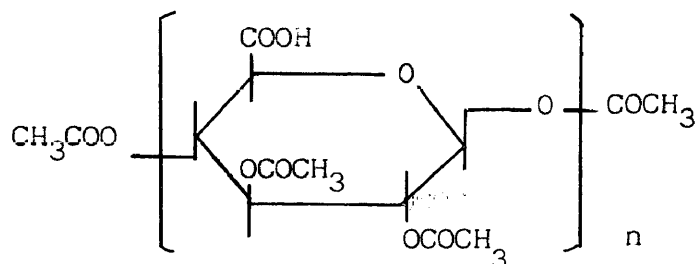
n	No. of acetyl groups	% CH$_3$CO	%C	%H	%O
2	4	32.0	44.7	4.8	50.7
	5	37.1	45.5	4.7	49.7
	6	42.4	46.3	4.7	48.9
3	6	32.4	45.1	4.8	50.1
	7	35.8	45.7	4.8	49.5
	8	39.9	46.3	4.8	48.9
4	8	32.6	45.4	4.7	50.0
	9	35.2	45.8	4.7	49.5
	10	37.6	46.2	4.7	49.2
large	completely acetylated	33.1	46.2	4.6	49.2

It may be seen that the analytical values for the acetylated enzyme hydrolyzate (45.4% C, 5.1% H, 49.5% O, 35.2% CH$_3$CO) would correspond to a product of the above structure which is not completely acetylated, e.g., a tri-glucuronic acid hepta-acetate or a tetra-glucuronic acid nona-acetate.

The analysis for nitrogen content of the derivative of the enzyme hydrolyzate with 2,4-dinitrophenylhydrazine provides a means of determining the molecular weight of the enzyme hydrolyzate. It must be assumed that no chemical hydrolysis of the enzyme hydrolyzate occurred during the reaction with 2,4-dinitrophenylhydrazine; in view of the mild reaction conditions involved, and considering the strength of acid needed to cause hydrolysis (cf. section IV-2), this assumption seems sufficiently well founded.

Analytical data calculated for a 2,4-dinitrophenylhydrazone of the following structure are given below:
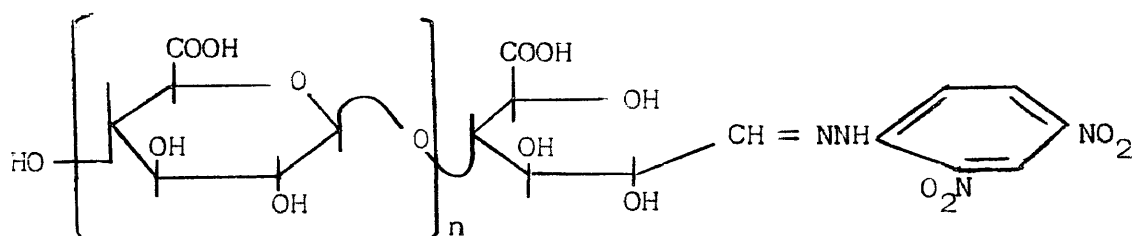
n	%C	%H	%O	%N
1	39.3	4.0	46.5	10.2
2	39.7	4.1	48.5	7.7
3	40.0	4.2	49.7	6.2
4	40.1	4.3	50.5	5.2

It may be seen that the analytical data for the derivative

(40.3% C, 4.9% H, 7.9% N) correspond approximately to $n = 2$, i.e.,

indicating that the enzyme hydrolyzate consists essentially of a

tri-glucuronic acid.

The use of these data for the estimation of molecular weight

is subject to some qualifications. There is a possibility that

more than one hydrazine group could be introduced into the molecule.

Therefore, strictly speaking, the nitrogen content determines only

the minimum molecular weight of the derivative and hence of the

enzyme hydrolyzate. Also the true structure of the derivative is

not known; the possibility of lactone formation exists, for example.

This would have the effect of increasing the nitrogen content. Thus,

the removal of one molecule of water from the formula pictured above

($n = 2$) gives calculated analytical values of 40.6% C, 4.0% H,

47.5% O, and 7.9% N.

Direct determination of the molecular weight of the enzyme

hydrolyzate by the freezing-point depression method was hampered by

the limited number of solvents for the enzyme hydrolyzate and its

derivatives. Furthermore, the accuracy of the method was low because

of the relatively large molecular weights of the materials under in-

vestigation.

This type of determination was carried out for the enzyme

hydrolyzate itself and for its reaction product with diazomethane,

supposedly the methyl ester of the enzyme hydrolyzate. The cryo-
scopic method could not be applied to the acetylated enzyme hydrolyzate
or to its derivative with 2,4-dinitrophenylhydrazine, because of the
lack of suitable solvents.

Using water as a solvent, the molecular weight of the enzyme
hydrolyzate was estimated from freezing point depression data as
800 ± 40. The discrepancy between this value and the lower values
derived from the chemical data may be due to the fact that, in pre-
paring the sample of the enzyme hydrolyzate for molecular weight
determination, one source of error may have been introduced while
removing another. As explained in section IV-11, it was necessary
to evaporate the sample repeatedly to dryness, finally drying over
phosphorous pentoxide, in order to remove adsorbed ethanol. This
treatment may well lead to lactonization or anhydride formation in-
volving the carboxyl groups of the glucuronic acid residues. This
would increase the molecular weight.

Alternatively, association in solution might be responsible for
the observed molecular weight. Therefore it was hoped that esteri-
fication of the carboxyl groups would remove both these possible
sources of error and permit an accurate determination of the
molecular weight by cryoscopy. The molecular weight of the methyl
ester of the enzyme hydrolyzate was estimated as 440 ± 20 using
water as the solvent. This value is roughly in agreement with the
molecular weight given by the chemical data.

The attempt to regenerate the enzyme hydrolyzate free acid
from its purified sodium salt by passage over a cation exchange

resin apparently led to some other changes in the molecule. This
is shown by the fact that the neutralization equivalent of the re-
generated material was higher than that of the original acid (230
compared with 185). It is possible that some lactonization, esteri-
fication or anhydride formation occurred as a result of catalytic
action of the ion exchange resin. The spontaneous lactonization
of glucuronic acid on regeneration from its salts is well known, and
is believed to involve a change from a pyranose ring in the free acid
or salt to a furanose ring in glucurono-δ-lactone[40].

Other evidence of structural change in the regenerated hydrolyzate
is given by the analytical data on the 2,4-dinitrophenylhydrazine
derivative made from the regenerated material. This derivative had
a nitrogen content of 12.6%, as compared with 7.9% for the same
derivative prepared from the original hydrolyzate.

A summary of the evidence which has been presented indicates
that the action of bacterial β-glucuronidase on celluronic acid
is the hydrolysis of the substrate to give an oligo-glucuronic acid
containing an average of about three glucuronic acid residues. These
data might be given by either a tri-glucuronic acid itself or by a
heterogeneous mixture of, for example, di-, tri-, and tetra-glucuronic
acids. Some evidence of homogeneity of the enzyme hydrolyzate is pro-
vided by the uniform crystalline nature of the sodium salt, as well as
the lack of separation of components on chromatographing the 2,4-
dinitrophenylhydrazine derivative.

The question then arises as to why the enzyme might give a fairly
uniform product containing three glucuronic acid residues, rather than

monomeric glucuronic acid, for example. Apparently no evidence has

ever been presented for the enzymatic hydrolysis of polyglucuronic

acids; in the analogous case of polygalacturonic acid (pectic acid),

however, the production of galacturonic acid by polygalacturonidase is

well known. Recently, the intermediate stages in this hydrolysis have

been studied. By the use of partition chromatography, the intermediate

di-, tri-, tetra-, and penta-galacturonic acids have been identified[33]

and the di- and tri-galacturonic acids isolated in pure condition.[41]

Since the chromatography of the celluronic acid enzyme hydrolyzate

(section IV-2) was carried out under conditions identical with those

used for the pectic acid hydrolyzate (i.e., isobutyric acid as solvent),

it is possible to compare the celluronic acid hydrolyzate with the

mixture of oligo-galacturonic acids obtained from pectic acid. The

distance moved by the streak of celluronic acid hydrolyzate (8 cm.)

compared with that moved by the glucuronic acid spot (22 cm.) is in

the same ratio as that found for a tri-galacturonic acid compared

with galacturonic acid[33].

In addition to the production of galacturonic acid by poly-

galacturonidase, evidence has also been presented for the breakdown

of pectic acid by a Neurospora enzyme ("depolymerase") to yield

low molecular weight polygalacturonic acids (minimum chain length

7-8 uronic acid units)[42]. The action of this enzyme is manifested

first by a rapid drop in the viscosity of sodium pectate solutions and

a loss of the colloidal properties of the substrate, followed by an

increase in the reducing value of the solution. There is also

evidence that polygalacturonidase itself may contain two enzymes,

one of which degrades pectic acid to di- and tri-galacturonic acids,
which are then hydrolyzed to galacturonic acid by the second enzyme.[43]

In the case of celluronic acid, a heterogeneous structure is
present, the chains of this substance consisting of glucuronic acid
and glucose residues in proportions depending on the extent of oxida-
tion. The sample of celluronic acid which was used in this
investigation had a glucuronic acid: glucose ratio of 4:1 (80%
oxidized). However, it must not be presumed that this implies a
regular structure in the polymer chain. Rather it is likely that
the extent of oxidation of the cellulose chain is dependent on the
accessibility of the glucose units to the oxidant, and it would
therefore be expected that the more accessible portions would be
completely oxidized to polyuronic acid. The less accessible portions
of the cellulose structure might be much less affected by the oxidant.
In this connection it may be noted that increasing oxidation of
cellulose with nitrogen dioxide leads to increasing loss of the
original fibrous structure.[8]

Therefore it is possible that the action of bacterial β-
glucuronidase on celluronic acid may be confined to those portions
of the substrate which consist essentially of polyglucuronic acid
chains. Animal β-glucuronidase is known to have no action on
the β-glucosidic linkage of cellobiose[24], and the experiment
described in section IV-13 showed that bacterial β-glucuronidase
is also inactive towards cellobiose. This indicates that the
enzyme would be unable to hydrolyze the β-glucosidic linkages
in celluronic acid, and would act only on the β-glucuronic acid

linkage between two glucuronic acid residues or that between carbon

one of a glucuronic acid residue and carbon four of a glucose residue.

The action of β-glucuronidase requires certain structural

features in the substrate. These include the β-linkage at carbon

one, the carboxyl of carbon six (i.e., distinction from β-glucosidase),

the glucose configuration of carbon three (distinction from polygalacturon-

idase), and the pyranose ring.[44] It may be suggested therefore that

the action of β-glucuronidase on celluronic acid involves the

formation of an enzyme-substrate complex in which positions one,

three and six as well as the pyranose ring of the glucuronic acid

residue are involved. Hydrolysis of the glycosidic linkage at

carbon one then occurs, followed by dissociation of the complex.

In order to explain why the enzyme does not lead to complete

hydrolysis of the celluronic acid, with the formation of glucuronic

acid, it may be supposed that in the enzyme-substrate complex, a

blocking action of the enzyme molecule prevents the close approach

of other enzyme molecules containing active reaction sites. Thus

if complex formation were restricted to every fourth glucuronic acid

residue, the product of enzymatic hydrolysis would be a tri-glucuronic

acid. An analogous mechanism may be operative in the above mentioned

production of oligo-galacturonic acids by "depolymerase" or poly-

galacturonidase, and in the hydrolysis of cellulose to cellobiose and

oligosaccharides by cellulase enzymes.[45]

That the enzyme hydrolyzate may be hydrolyzed to glucuronic

acid by 80% sulfuric acid is suggested by the chromatograms in

Figure 3. The effect of the acid on the molecular weight of the

enzyme hydrolyzate is shown by a comparison of B, the hydrolyzate itself, with C, the acid treated hydrolyzate. The products resulting from the action of the sulfuric acid were not identified. The heterogeneous nature of celluronic acid is reflected in chromatogram D, as compared with the simpler results of the acid hydrolysis of pectic acid (E).

The production of oligo-glucuronic acids from celluronic acid, as observed in this investigation, suggests that the absorption of celluronic acid gauze in the animal body may occur by this means. The product, with a molecular weight much lower than that of celluronic acid, could then be transported through the body for elimination or might serve as the basis for metabolic processes.

VI   SUMMARY

1. Solutions of bacterial β-glucuronidase cause the dissolution of nitrogen dioxide oxidized cellulose (celluronic acid, mainly 1,4-polyanhydroglucuronic acid), and the same enzyme causes a rapid decrease in the viscosity of solutions of partially neutralized celluronic acid.

2. These data are interpreted to mean that the β-glucuronidase catalyzes the hydrolysis of the celluronic acid into low molecular weight oligo-glucuronic acids, which were isolated from the reaction in 30-50% yield.

3. The absence of appreciable amounts of glucuronic acid in the hydrolysis products was demonstrated.

4. From the purified enzyme hydrolyzate, a barium salt, a crystalline sodium salt, an acetate and a methyl ester were obtained and characterized.

5. The derivative which was obtained from treatment of the enzyme hydrolyzate with 2,4-dinitrophenylhydrazine gave analytical results indicating that the enzyme hydrolyzate consisted mainly of a tri-glucuronic acid.

6. The cryoscopic molecular weight determination on the enzyme hydrolyzate methyl ester was in agreement with this result, as were the analytical data on the barium and sodium salts.

7. Possible mechanisms of the enzymatic hydrolysis of celluronic acid are discussed in the light of the structure of celluronic acid.

8. It is suggested that enzymatic hydrolysis by β-glucuronidase may be involved in the absorption of nitrogen dioxide oxidized cellulose gauze in body cavities.

VII   BIBLIOGRAPHY

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