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STRUCTURAL STUDIES
ON THE
POLYSACCHARIDE MATERIAL
OF THE
HYDATID CYST MEMBRANE

BY

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submitted in partial fulfillment for the requirements
of the degree Master of Science
in the Chemistry Department of the
American University of Beirut
Beirut, Lebanon
February 1966

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This work is dedicated to Z.

ACKNOWLEDGMENT

It is with great pleasure and gratitude that the author acknowledges the encouragement and conscientious support of Professor Shibli I. Bayyuk who directed this study.

He also wishes to thank Dr. C. Schwabe for the supply of hydatid cyst membranes, Mr. D.J. Palmer for the generous gift of bee venom, Mr. R. Guy for the determination of the infrared spectra, Mr. S. Ishak for facilitating the laboratory work and Mr. W. Read for extending instrumental facilities.

Thanks are also due to the Rockefeller Foundation and to the Arts and Sciences Research Committee for financial support of this investigation.

ABSTRACT

The polysaccharide components of the whole hydatid cyst membrane were isolated, purified, and their structures examined. Only two polysaccharides could be detected. The minor component, identified as glycogen, had a high positive rotation and was present in minute concentrations firmly bound to proteins. The origin of this fraction, whether from the endocyst or ectocyst or both, could not be ascertained.

The major polysaccharide was electrophoretically-homogeneous, alkali-labile, and had $[\alpha]_D = +4.2^\circ$. It consisted of equimolar amounts of D-galactose and N-acetylhexosamine. The hexosamine moiety was found to consist of D-galactosamine and D-glucosamine in the ratio 4:1. The anomeric linkages were shown to be of the β -type as evidenced from infrared spectroscopy, optical rotation measurements and enzymatic hydrolysis. The galactose and N-acetylhexosamine units might be alternating in the polysaccharide chain, but further evidence is necessary.

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INTRODUCTION

Purpose of the work

Available information about the structure of the polysaccharide component of the hydatid cyst membrane is fragmentary and incomplete. This is mainly attributable to the fact that little work, often employing crude techniques, has been done on the structure of this polysaccharide material; and secondly, no systematic isolation and structural study have been attempted.

The purpose of the present investigation was to isolate the polysaccharide material under the mildest possible conditions, to purify it, to examine its homogeneity and to study its structure by classical methods used in carbohydrate chemistry.

Historical

Hydatid disease (hydatidosis, echinococcosis) is generally a health problem in many sheep- and cattle-raising countries such as Australia, New Zealand, Uruguay and Turkey. The Middle East is also considered as an endemic area^{1,2} in which the rate of incidence is believed to be one of the highest in the world.² Information about the prevalence of human hydatidosis in this area is deficient in a number of respects, and due to the lack of reliable statistics, the actual rate of infection is much higher than the published figures reveal.^{1,3} Available data, as incomplete as they may be, still

indicate the wide-spread occurrence of the disease.

During the period 1923 - 1949, hospital records in Lebanon, Syria, Iraq and Palestine indicated that a total of 385 human cases were admitted for treatment.¹ Schwabe and Abou Daoud² studied the epidemiology of human echinococcosis in Lebanon during the period 1949 - 1959, and found that the annual rate of surgically confirmed cases was 3.82 per 100,000 population, which is one of the highest reported. In a more recent study³ the risk of infection with hydatid disease in Beirut was found to be closely associated with dog ownership.

The adult stage of Echinococcus granulosus (about 2 - 8 mm. long) usually lives in the intestine of the dog, wolf or fox. It consists of a scolex and neck followed by three or four segments. The last segment is usually mature or nearly so and contains about 500 - 800 eggs each of which, under proper conditions, may eventually develop into a cyst. The last segment, before or after rupturing, gets detached and the contents excreted with the feces of the definitive host. After dispersal, ova gain access to the gastrointestinal tract of the intermediate host (pig, horse, rabbit, sheep, cattle) through contaminated food or water. There the ovum hatches and the liberated larva pierces through the intestinal wall into a blood vessel and is carried by the blood stream until it finally lodges in one of the organs where the blood vessels become too narrow to allow its continued passage. The larva then develops into a hollow bladder that imbibes a large volume of fluid from the host. The host's organ reacts by building a fibrous cyst wall around the bladder.

The size of the cyst thus formed may reach the size of a tennis-ball or even larger. In man, 60 to 75% of the larvae grow in the liver and around 20% in the lungs. The chance of a cyst developing in other organs (kidney, heart, brain, muscles, bone) is low.⁴

The cyst wall consists of an inner germinal membrane (endocyst) which functions for permeability control and osmoregulation, and an outer non-adherent non-living laminated membrane (ectocyst).⁵ The endocyst is composed of a granular substance presenting a cellular structure and gives rise to the brood capsules and scolices. The ectocyst consists of many translucent structureless laminae. The laminated membrane is believed to result from the interaction of a mucopolysaccharide produced as a metabolic product of scolices with some unknown host component.

The first observation on the nature of the polysaccharide constituent of the cyst membrane was made by Schmiedeberg⁶ who reported the presence of a polysaccharide-protein complex which, upon hydrolysis with concentrated H_2SO_4 at 110° , gave glucosamine and a hexose. Schmiedeberg thought that the polysaccharide material was related to chitin or chondroitin. Flössner⁷ confirmed the presence of glucosamine by preparing the anhydride of glucosamine phenylisocyanate (m.p. 209° ; for $C_{13}H_{16}N_2O_5$ calc. N = 10.0%, found 10.36%). He furthermore suggested that glucosamine probably was the main, if not the only, reducing substance.

In a study of the staining reactions of hydatid cysts, Cameron⁸ found that, while glycogen was distributed throughout the entire cyst wall, the endocyst contained larger concentrations than did the ectocyst.

According to Brault and Loeper⁹, however, glycogen was found to be present only in the germinal membrane. In a more elaborate investigation of the principal organic and inorganic constituents of the cyst wall by micro-chemical methods, Fitzpatrick and Cameron¹⁰ obtained a positive reaction to both Langhans' and Best's tests, thus confirming the presence of glycogen throughout the whole cyst wall. Later, Kilejian and coworkers¹¹ reported that the germinal membrane contained glycogen deposits, but the laminated membrane contained minute amounts of glycogen or none at all.

The first attempt on the elucidation of the nature of the polysaccharide component of the cyst wall by actual isolation was made by Cmelik.¹² He extracted the material from the membranes by four different methods:

- a. extraction with water at a pH of 3.5,
- b. 5% trichloroacetic acid extraction after Boivin,¹³
- c. digestion of the proteins with pepsin (pH 4),
- d. extraction by combined proteolytic digestion with pepsin (pH 4) and trypsin (pH 7.8).

The first three methods gave impure preparations; the last method, however, yielded a protein- and nucleic acid-free antigenic material which he called "substance D". It gave a positive Molisch test, a positive Dische reaction for deoxypentoses and, after hydrolysis, a positive Elson-Morgan reaction for amino sugars. The major products were reported to be glucosamine and aldohexoses, while ketoses and pentoses were found to be absent.

Recently, Kilejian and coworkers¹⁴ investigated the polysaccharide material present in the laminated membrane further. According

to their findings only one polysaccharide consisting of galactose and glucosamine as building units seemed to be present. It was also observed that this polysaccharide occurred firmly bound to proteins, and drastic conditions were necessary to liberate the polysaccharide moiety in the free state. It was noticeable that no glycogen was present in the laminated membrane as evidenced by the absence of glucose in acid hydrolyzates of the polysaccharide component.

It is obvious that even some of this scanty information about the polysaccharide constituent of the cyst membrane is still lacking experimental evidence. This prompted the initiation of the present work.

RESULTS AND DISCUSSION

In this study only membranes of Echinococcus granulosus cysts obtained from infected cattle and sheep were used. The cysts were cut open, freed of cyst fluid, and the membranes obtained were rinsed with water and stored at -25° until an appropriate amount was collected. This ensured inhibition of possible enzymatic activity prior to extraction.

Extraction

As a principle, to effect minimum degradation of the polysaccharide component, neutral solutions should be used in the extraction procedure, and the process must be carried out at a low temperature. Acidic or basic solutions, as well as drastic conditions, usually result in degraded material. It is quite noticeable that previous workers performed their extractions under unfavorable conditions to the effect that they could not isolate the polysaccharide material without appreciably degrading it. In order to isolate the component in a form that represents as closely as possible the actual state in which it occurred in the membranes, a very mild extraction procedure was adopted.

The membranes were homogenized and extracted at 4° first with acetone and then with methanol-chloroform (3:1 v/v) to remove lipids and coloring matter. The polysaccharide constituent of the defatted

dry tissue was isolated by extraction with toluene-saturated 2 M - NaCl (to inhibit bacterial growth) at 4⁰, and the saline extract was deproteinized by repeated shakings with chloroform-amyl alcohol (9:1 v/v) according to Sevag's¹⁵ method (Fig. 1). Upon dialysis and freeze-drying, 0.4650 g. of polysaccharide material (0.93% of defatted dry tissue) was recovered and called Extract I. Obviously, this fraction consisted of the polysaccharide component which occurred either free or weakly bound to proteins.

Due to the fact that polysaccharides, especially acidic polysaccharides, also occur in firm combination with proteins in tissues, the residue left after the saline extraction was further extracted with N - NaOH for 24 hours at room temperature. The extract, after neutralization with glacial acetic acid, was deproteinized by the Sevag method, dialyzed against running tap water and lyophilized. The polysaccharide component obtained weighed 0.3227 g. (1.09% of residue tissue) and was designated as Extract II (Fig. 1). This fraction represented the polysaccharide constituent that occurred in firm combination with proteins.

Examination of the Purity of Extracts I & II

The usual contaminants of tissue polysaccharides are nucleic acids and proteins. To detect any such contamination, the ultraviolet absorption spectrum of each polysaccharide fraction was determined in the region 200 - 420 μ . The spectra of both fractions (Fig. 2) revealed the absence of protein (λ_{max} around 275 μ) and nucleic acid (λ_{max} around 260 μ) contaminants. Further evidence for the purity of Extracts I & II

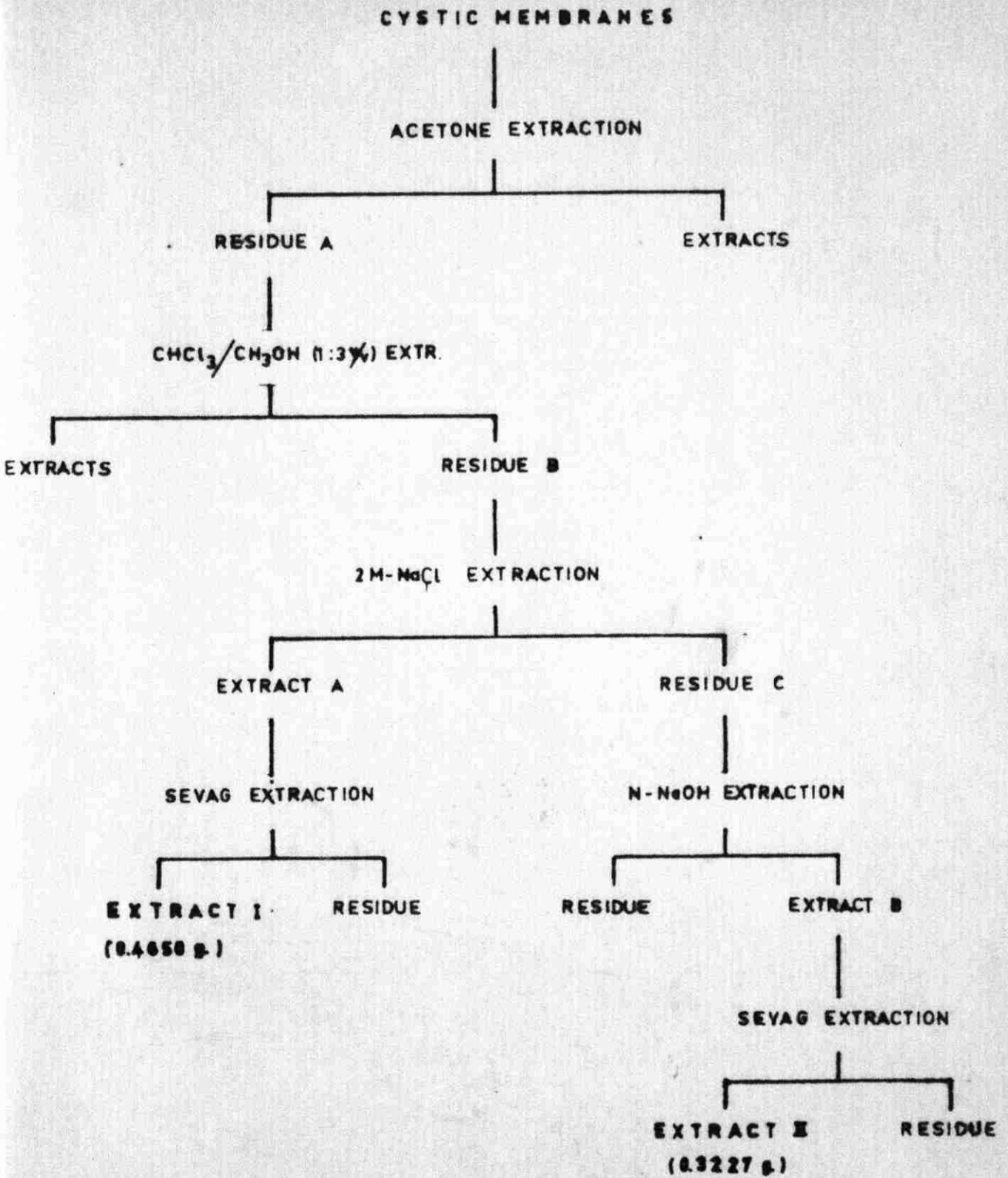


FIG.1 SCHEMATIC REPRESENTATION OF
EXTRACTION PROCEDURE A

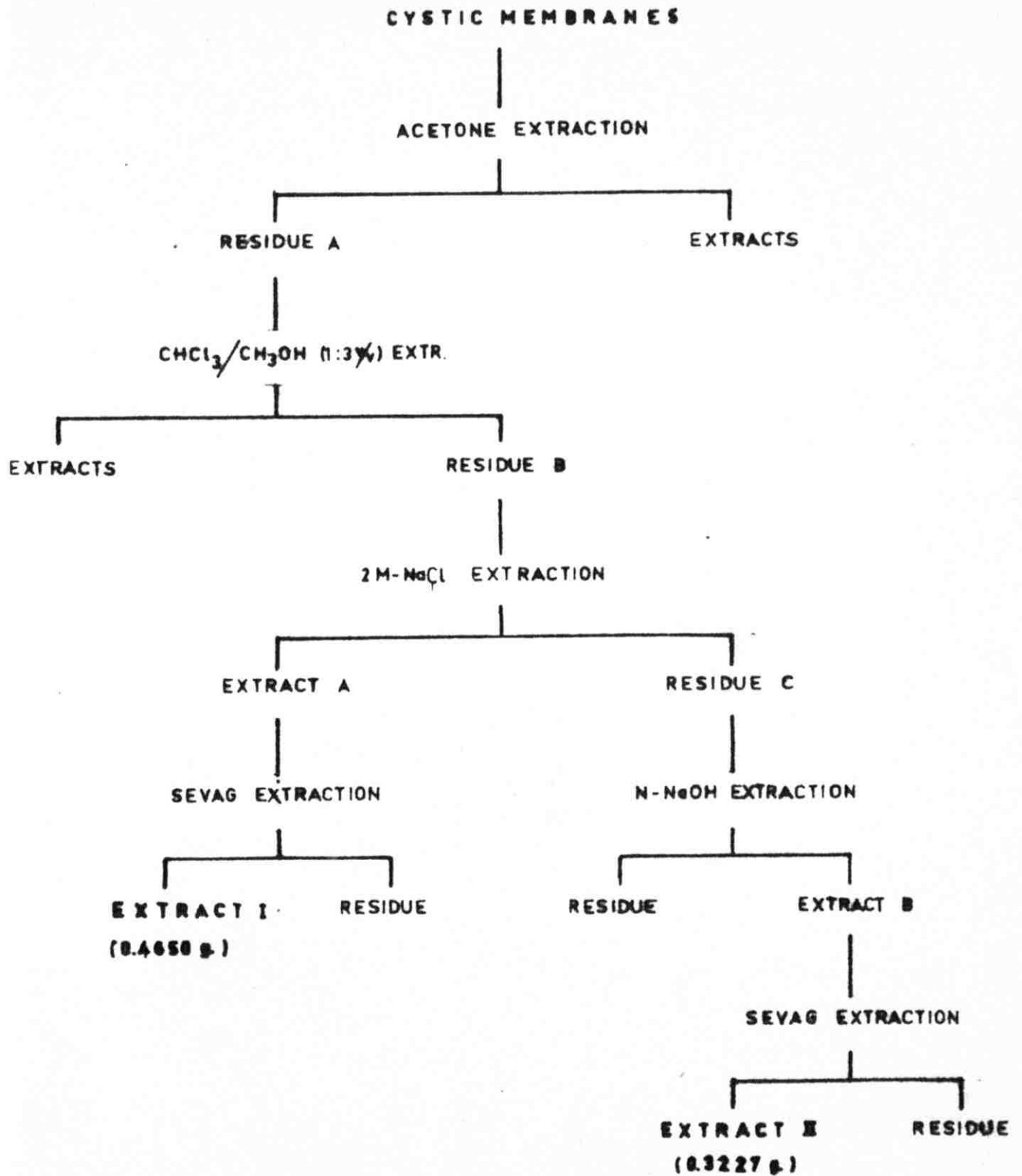
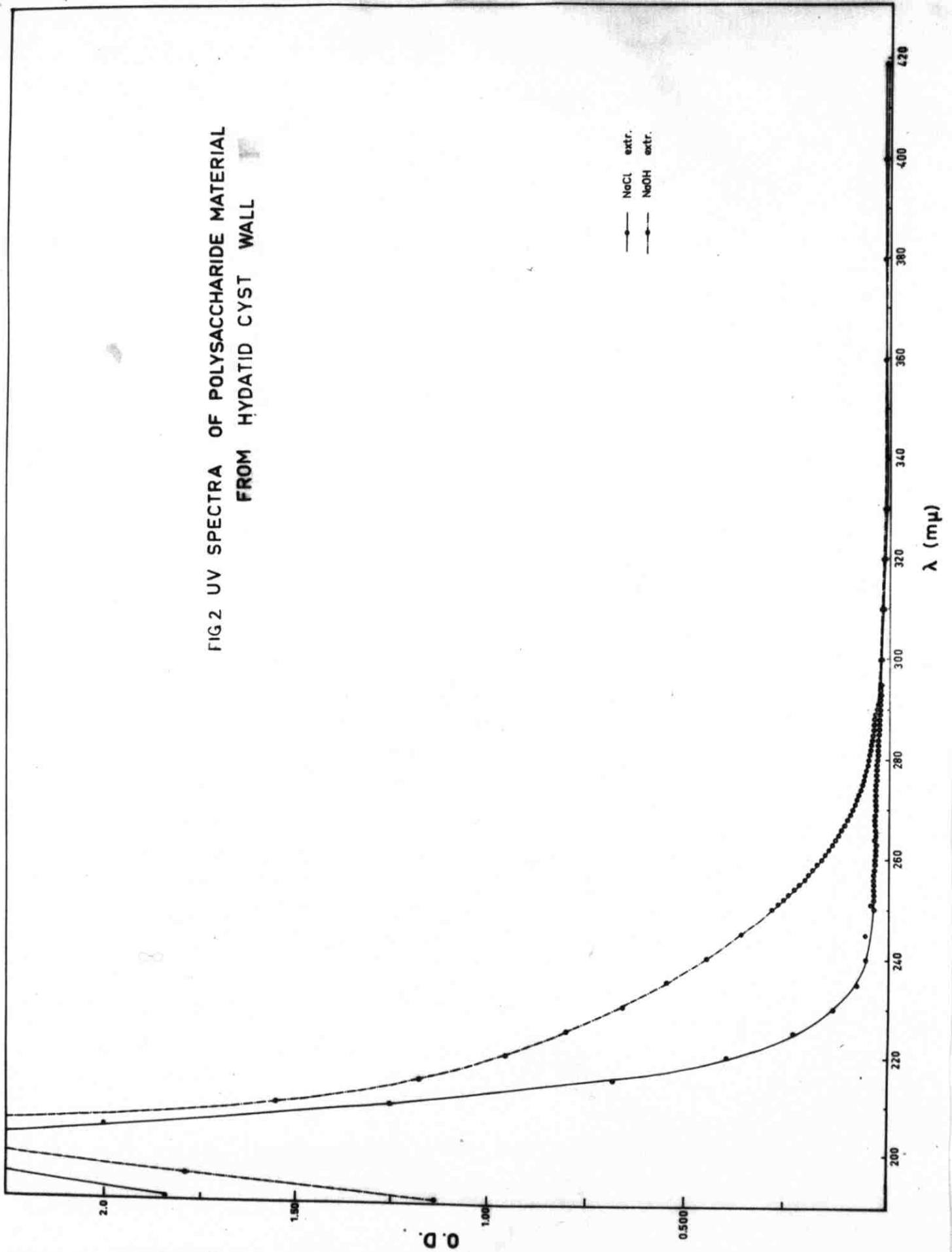


FIG.1 SCHEMATIC REPRESENTATION OF
EXTRACTION PROCEDURE A

FIG 2 UV SPECTRA OF POLYSACCHARIDE MATERIAL FROM HYDATID CYST WALL



was obtained by paper chromatographic examination of the acid hydrolyzates of both fractions. No peptides or amino acids could be detected.

Criterion of Homogeneity

Several physico-chemical methods are used for determining the homogeneity of a polysaccharide. These include:

- (i) fractional solubility
- (ii) diffusion
- (iii) study of the sedimentation pattern in an analytical ultracentrifuge^{16,17,18}
- (iv) electrophoretic mobility of the polysaccharide using different buffers
- (v) serological activity in case the polysaccharide is biologically active.¹⁹

The first method is rather crude, and due to certain instrumental limitations, electrophoresis was chosen to give evidence for any lack of homogeneity of the polysaccharide fractions. Upon examination of the electrophoretic mobility of Extracts I & II on paper using 0.1 M - borate buffer²⁰ (pH 9.20), the following observations were made:

- a) Each fraction moved as a single band. This indicated homogeneity and the great possibility that Extracts I & II consisted of one component each.
- b) The polysaccharides of Extracts I & II had identical mobility which suggested that they could be similar if not identical.

Contrary to the findings of previous workers¹⁴, it was evident that at least half of the membrane polysaccharide occurred either free or in loose combination with proteins; the rest seemed to be firmly bound to proteins and drastic conditions (treatment with N - NaOH at room temperature) were necessary to break the polysaccharide-protein linkage.

Infrared Spectra of Extracts I & II

The infrared absorption spectra of both polysaccharide fractions (Fig. 3) were determined in potassium bromide discs.²¹ Both polysaccharides exhibited strong absorption in the 3200 - 3400 cm^{-1} region attributable to O - H and N - H stretching, and weaker absorption at about 2900 cm^{-1} due to C - H stretching.²² Both absorbed at 1630 - 1645 cm^{-1} (C = O stretching), at 1525 - 1550 cm^{-1} (N - H deformation) and at 1380 - 1385 cm^{-1} (CH_3 - group); these are indicative of N-acetyl-amino group.^{23,24} The weak bands in the regions 1320 - 1330 cm^{-1} and 1230 - 1235 cm^{-1} are associated with amide and acetyl groups respectively.²⁵ Absorption peaks in the less characteristic region 1000 - 1220 cm^{-1} , are attributable to C - O stretching and C - O - H bending modes.²⁶ In the fingerprint region (700 - 1000 cm^{-1}), absorption at 875 - 880 cm^{-1} and complete absence of absorption at $844 \pm 8 \text{ cm}^{-1}$ are indicative of β -anomeric linkages.²³

It is outstanding to note that both spectra gave a clear evidence for the absence of sulfate groups (absorption at 1230 - 1250 cm^{-1}) and unionized carboxylic acid and lactone groups (absorption at 1735 cm^{-1}), which indicated that both polysaccharides were neutral (no uronic or

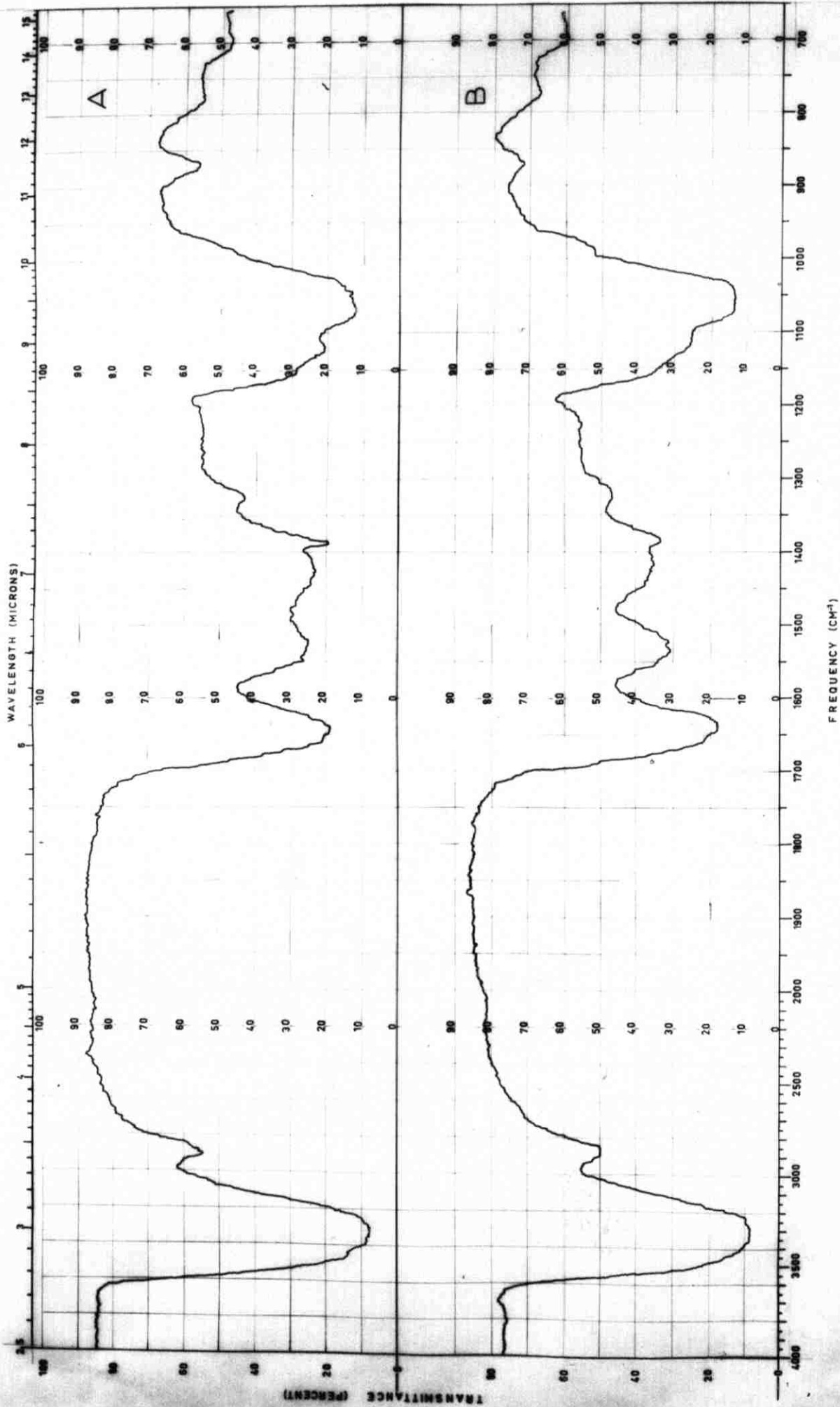


FIG. 3 INFRARED SPECTRA OF POLYSACCHARIDE ISOLATED FROM HYDATID CYST WALL FOLLOWING NaCl EXTRACTION (A); FOLLOWING NaOH EXTRACTION (B)

sulfuric acid groups). Further evidence, however, is still necessary to substantiate this conclusion. Furthermore, absence of absorption at 925, 758 and 845 cm^{-1} indicated the absence of glycogen. The spectra also gave further evidence for the fact that Extracts I & II were very closely similar, if not identical, in structure; both contained an acetyl hexosamine building unit and both had β -glycosidic linkages.

Preliminary Identification of the Monosaccharide Units

The monosaccharide building units of Extracts I & II were identified by paper chromatographic analysis of acid hydrolyzates (2 N - H_2SO_4 for 4 hours at 100°) using n-butanol - acetic acid - water (4:5:1 v/v)^{27,28} as developer. Both polysaccharides were found to be composed of the same monosaccharide residues - galactose and a hexosamine with a mobility similar to that of glucosamine (Fig. 4). It was obvious from both chromatograms that glucose and uronic acids were completely absent, thereby excluding the presence of glycogen and ruling out the presence of a uronic-acid-containing mucopolysaccharide.

It is interesting to note that Extracts I & II are of the very few tissue polysaccharides that do not contain acidic functional groups.

Identification of Glycogen

It is well known that some tissue glycogen occurs in very firm combination with proteins and drastic alkali treatment is required for its extraction. To find whether any glycogen of this type was present in the cyst membranes, it was decided to perform another extraction

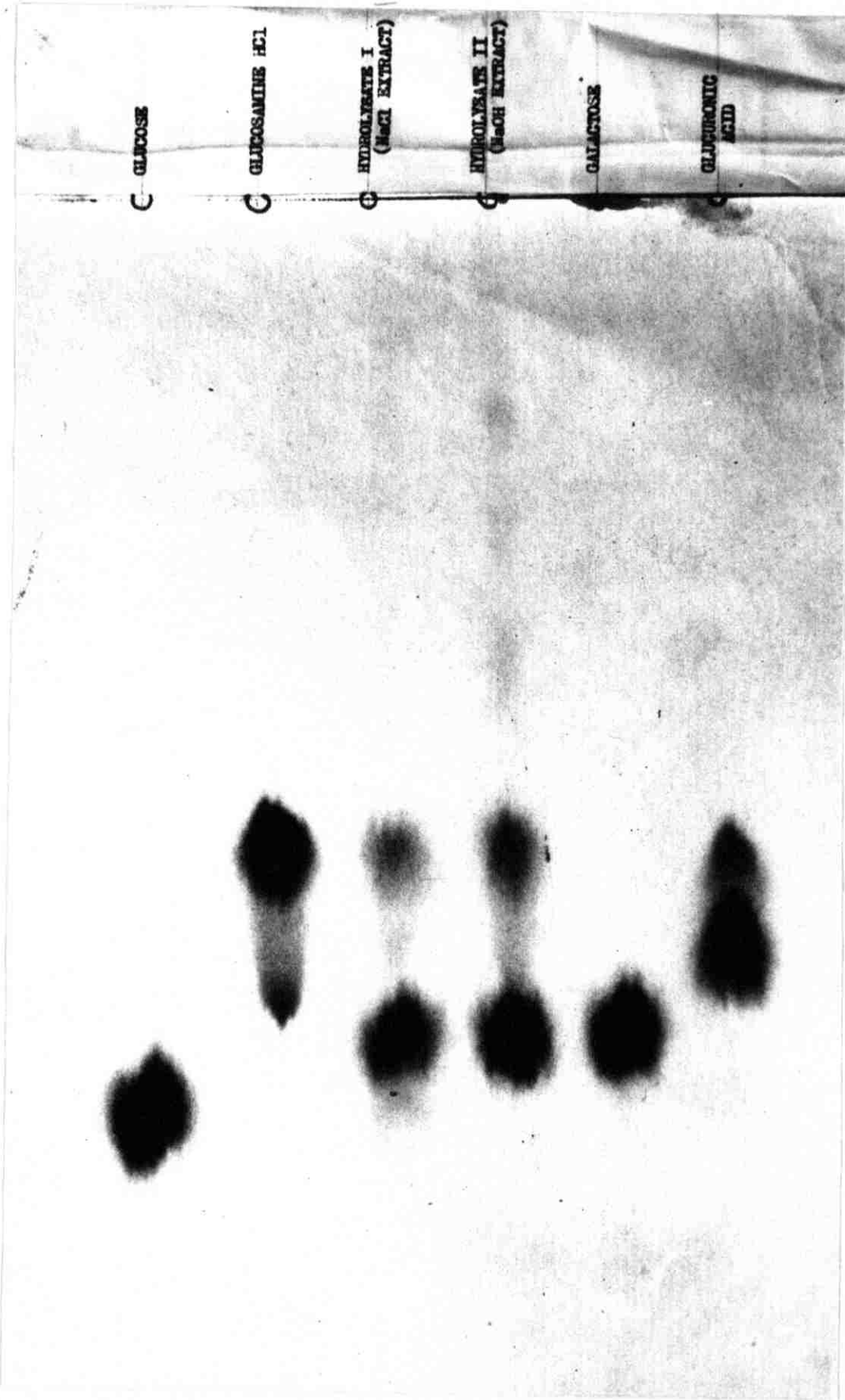


Fig. 4. Paper chromatogram of acid hydrolyzates of Extracts I & II (Solvent: n-butanol - acetic acid - water, 4:1:5 v/v).

according to Pflüger's technique²⁹ since saline and N - NaOH treatments failed to extract any glycogen. 20.0 g. of the defatted dry cyst membranes were extracted with 30% KOH at 100° for 1 hour. The polysaccharide material finally recovered was designated as Extract III (Fig. 5) and weighed only 10.8 mg. (0.054% of defatted dry tissue).

Extract III was examined by paper chromatographic analysis of an acid hydrolyzate. Two solvent systems ethyl acetate - pyridine - water (2:1:2 v/v)³⁰ and n-butanol - pyridine - water (9:5:8 v/v)³¹ were used as developers. In both cases a glucose spot was identified, in addition to ^{those of} galactose and hexosamine (Fig. 6). Glucose here could well be a hydrolysis product of small amounts of glycogen. Determination of optical rotation of Extract III gave $[\alpha]_D = +225^{\circ}$. Such a high positive value together with the identification of glucose as a building unit imply the presence of glycogen.

At this stage two facts became quite clear:

a) Glycogen occurred in minute concentrations and was very firmly bound to proteins. This is supported by the findings of Kilejian et al.¹⁴ The origin of glycogen, whether from the endocyst or ectocyst or both, cannot be predicted since whole cystic membranes were extracted.

b) The major polysaccharide constituent (composed of galactose and hexosamine) was alkali-labile as evidenced from the very low yield obtained following Pflüger's extraction.

Evidence for the Presence of N-acetylamino Group

Further proof for the presence of N-acetylamino group in polysaccharides I & II was obtained by reacting both with the Morgan-Elson reagents

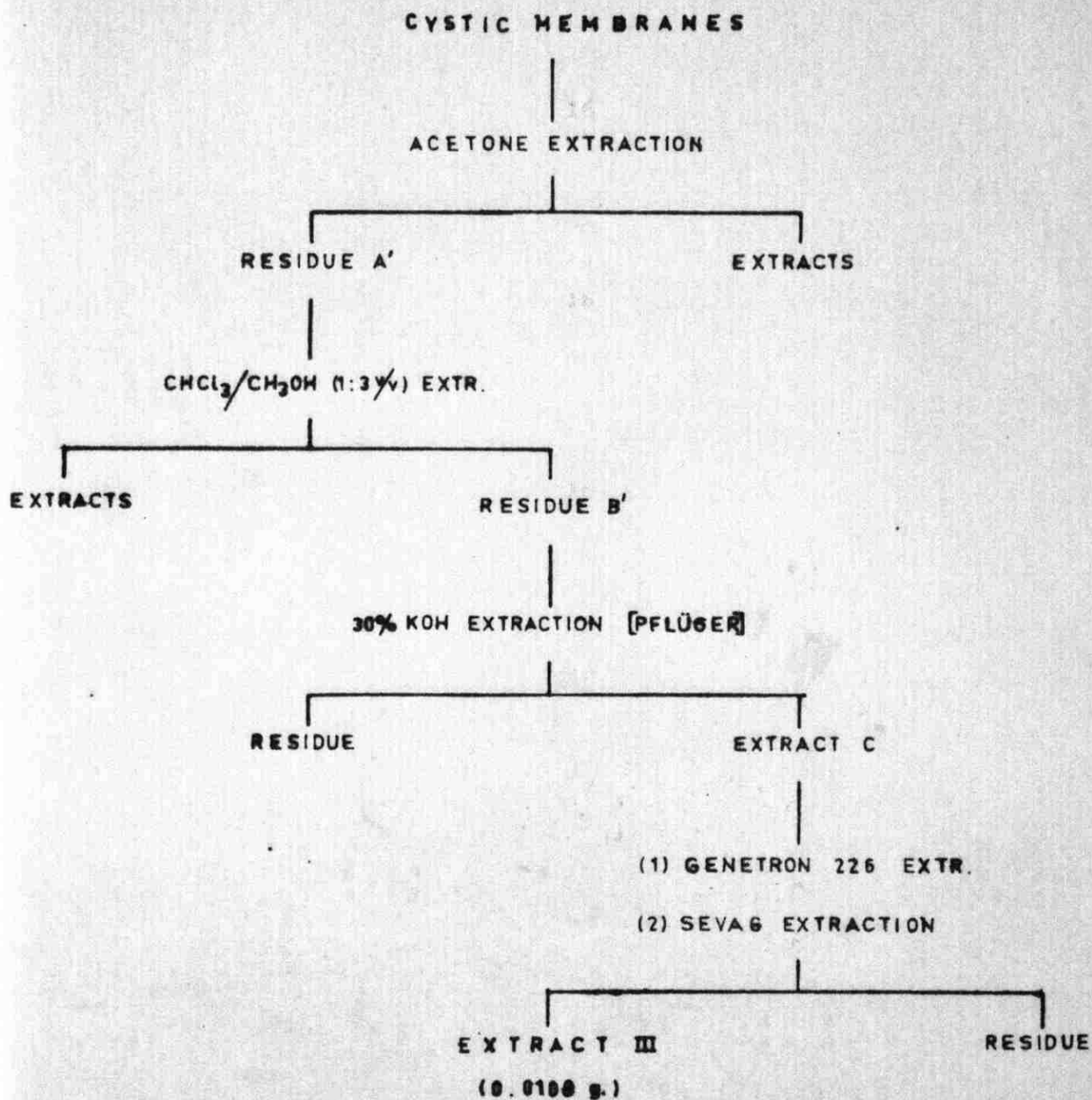


FIG. 5 SCHEMATIC REPRESENTATION OF
EXTRACTION PROCEDURE B

CYSTIC MEMBRANES

ACETONE EXTRACTION

RESIDUE A'

EXTRACTS

$\text{CHCl}_3/\text{CH}_2\text{OH}$ (1:3 v/v) EXTR.

EXTRACTS

RESIDUE B'

30% KOH EXTRACTION [PFLÜGER]

RESIDUE

EXTRACT C

(1) GENETRON 226 EXTR.

(2) SEVAG EXTRACTION

EXTRACT III

(0.0100 g.)

RESIDUE

FIG. 5 SCHEMATIC REPRESENTATION OF
EXTRACTION PROCEDURE B

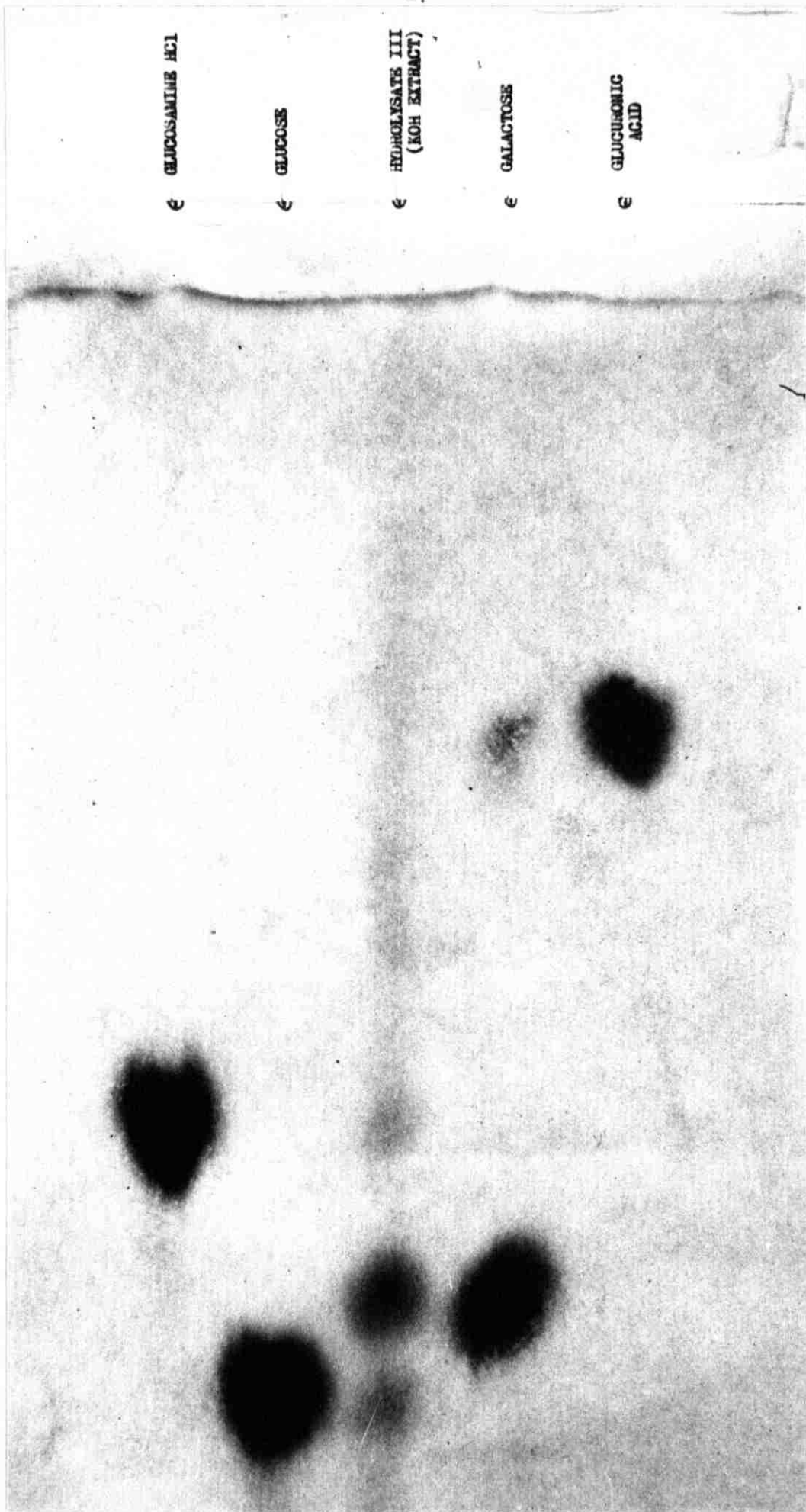


Fig. 6. Paper chromatogram of acid hydrolyzate of Extract III (Solvent: n-butanol - pyridine - water 9:5:8 v/v).

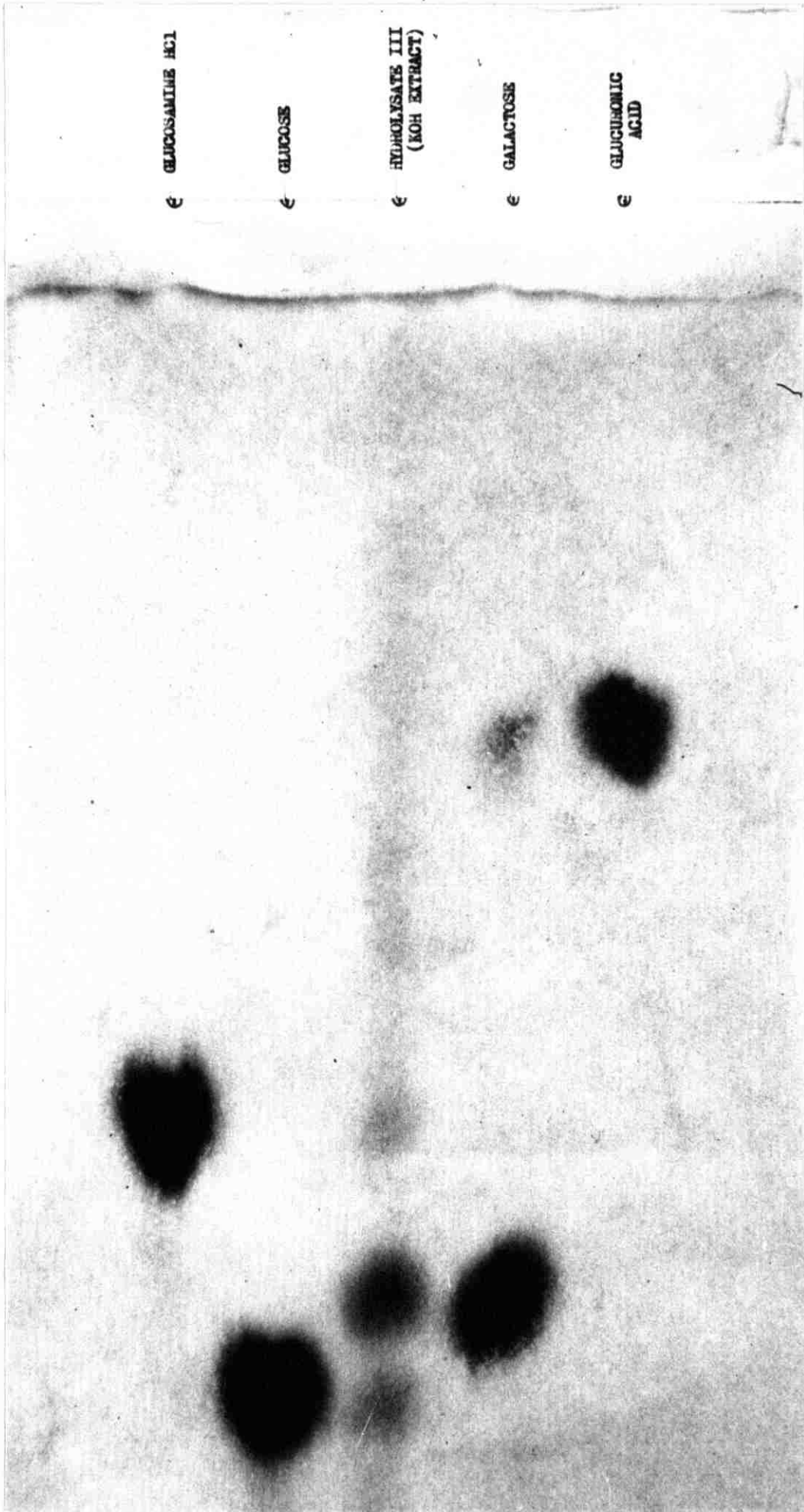
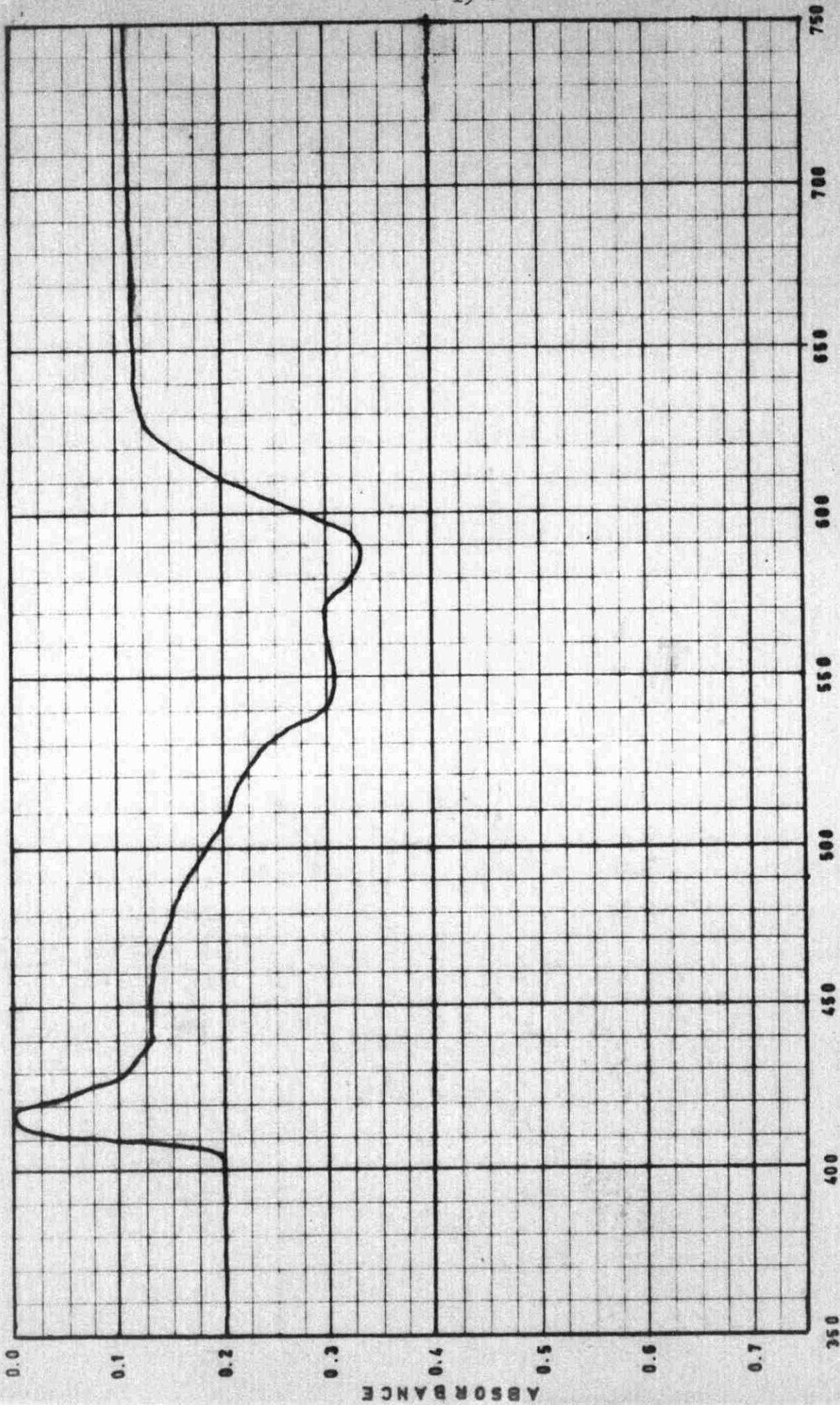


Fig. 6. Paper chromatogram of acid hydrolyzate of Extract III (Solvent: n-butanol - pyridine - water 9:5:8 v/v).



WAVELENGTH (MILLIMICRONS)

FIG. 7 ABSORPTION SPECTRUM OF COLOR
IN MORGAN - ELSON REACTION

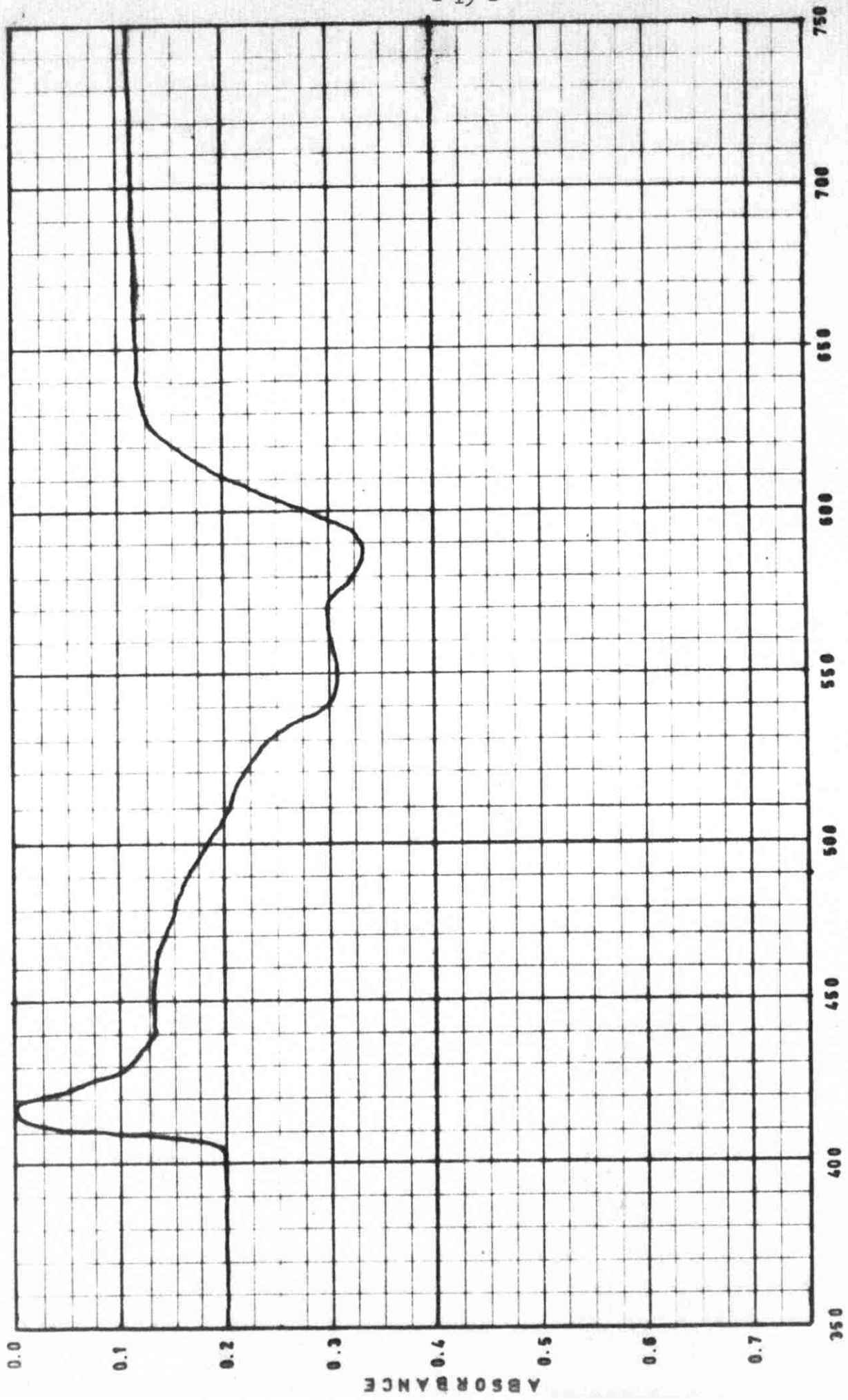


FIG. 7 ABSORPTION SPECTRUM OF COLOR
IN MORGAN - ELSON REACTION

absorption maximum at 535 μ .³⁶

The reaction is based on the formation of 5-carboxy-2-formylfuran (IV) which appears to be the chief chromogen responsible for color development



IV

in the presence of concentrated sulfuric acid.³⁷ The absorption follows Beer's law in the range 5-100 γ hexuronic acid per ml. and can be used for quantitative determination.³⁶ Hexoses, however, produce a brown-red color with a completely different absorption spectrum.³⁶ In quantitative determinations, the absorption due to hexoses which amounts to between 5 - 7% of that of glucuronic acid should be considered.

The following table shows the results obtained by performing the carbazole reaction on polysaccharide Extracts I & II, glycogen and hyaluronic acid.

Solution	Substance	Concentration (% solution)	O.D. at 535 μ
1	Potassium hyaluronate	0.02	0.332
2	Glycogen	0.02	0.148
3	Extract I	0.05	0.122
4	Extract I	0.02	0.063
5	Extract II	0.05	0.111
6	Extract II	0.02	0.047

Table 1. Dische's reaction for hexuronic acids

The above results provide straightforward evidence for the complete absence of hexuronic acids in both polysaccharide Extracts I & II, since on the basis of equal concentrations (solutions 4 and 6), the optical density value developed by a uronic acid-containing polysaccharide would be expected to lie between those of glycogen and hyaluronic acid.

Quantitative Determination of Galactose

Quantitative determination of the neutral sugar galactose in Extract I was performed according to Svennerholm's modification³⁸ of Vasseur's method.³⁹ To determine the optimal time for the preliminary hydrolysis, the polysaccharide was heated with 3 N - H₂SO₄ for different periods ranging between 2 - 6 hours in a boiling-water bath. The resulting hydrolyzates when treated with the orcinol reagent (orcinol in 98% H₂SO₄) produced a dark red color. The optical densities were read at 505 mμ against a sample blank and reference standards. The results obtained are tabulated below:

Time of hydrolysis (hours)	% D-galactose
2	40.5
3	54.0
4	43.0
5	40.5
6	26.2

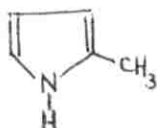
Table 2. Orcinol - H₂SO₄ reaction for D-galactose

Prolonged hydrolysis resulted in destruction of the neutral sugar.

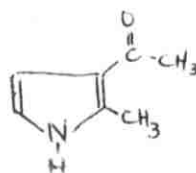
Quantitative Determination of the Hexosamine

The Elson-Morgan reaction as modified by Rondle and Morgan⁴⁰ was applied for the determination of the hexosamine content. This involved treatment of the free sugar with alkaline 2,4-pentanedione (acetylacetone), followed by reacting the resultant chromogens with Ehrlich's reagent (N,N-dimethyl-p-aminobenzaldehyde in acid solution) to produce a red color (absorption maximum 530 m μ). The extinction coefficient of the color produced by D-galactosamine is about 90% of that produced by D-glucosamine.

Little is known about the nature of the chromogens formed upon heating the hexosamine with 2,4-pentanedione in an alkaline medium. Of the chromogens suggested the following have been isolated and characterized:⁴¹



2-methylpyrrole (steam-volatile)



3-acetyl-2-methylpyrrole (not volatile with steam)

Other chromogens have been reported but no attempts to isolate pure compounds have been forthcoming.

Since the determination ought to be carried on the free hexosamine, it was necessary, at first, to find the optimal conditions for maximum

liberation and minimum destruction of the hexosamine. The results obtained with different values of the variables are given in the following table:

Hydrolysis period (hours)	% Aminosugar in Extract I		% Aminosugar in Extract II	
	3N-HCl, 105 ⁰	4N-HCl, 100 ⁰	3N-HCl, 105 ⁰	4N-HCl, 100 ⁰
1	18.5	--	--	--
2	18.8	--	--	--
3	19.5	--	--	--
4	24.9	--	23.0	--
6	21.1	--	27.6	22.9
8	19.7	--	22.8	19.9
10	16.9	20.2	20.8	20.8
15	--	17.8	--	--
18	--	15.9	--	--

Table 3. Effect of different hydrolytic conditions on aminosugar determination (calculated on the basis of 80% galactosamine and 20% glucosamine).

The optimal conditions for maximum liberation and limited destruction of the aminosugar in Extract I were 4 hours hydrolysis with 3 N - HCl at 105⁰, whereas the corresponding values for Extract II were 3 N - HCl at 105⁰ for 6 hours.

It has been recognized⁴² that whereas nitrogen-free neutral polysaccharides liberate, on acid hydrolysis, their constituent monosaccharide residues in yields close to theoretical values, those containing aminosugar do not. In such cases hydrolysis may proceed through one of two

pathways:

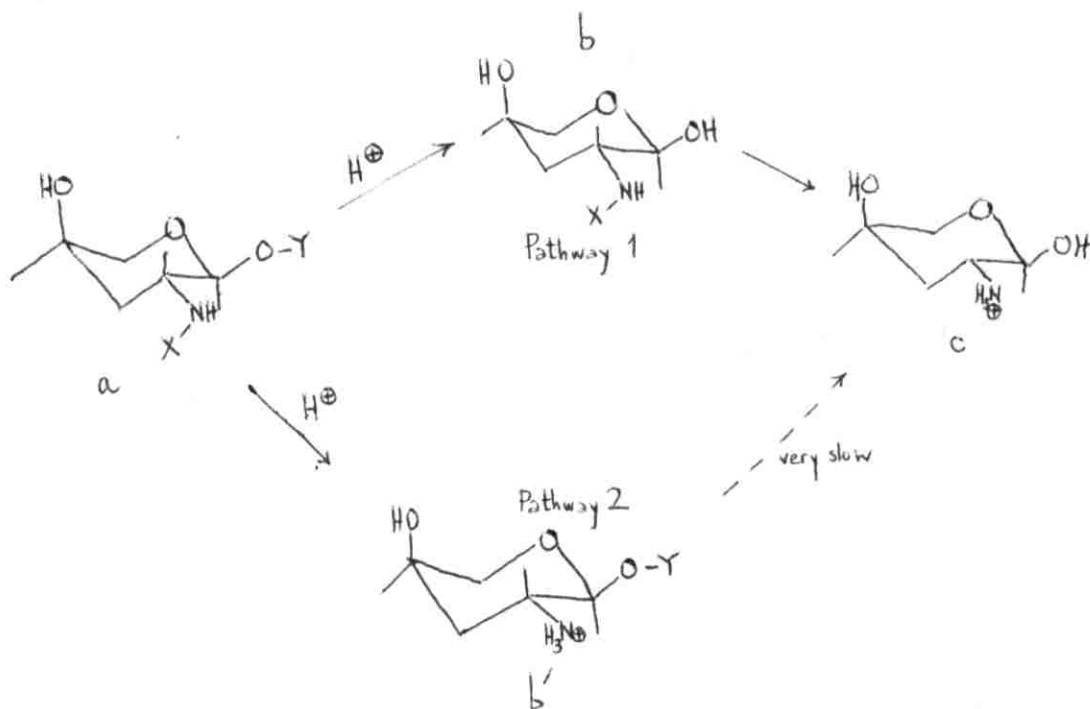


Fig. 8. Schematic representation of the acidic hydrolysis of D-galactosamine derivatives (Y = interglycosidic linkage, X = acetyl group).

If hydrolysis at the X - NH site follows that of the glycosidic substituent Y, the reaction is tunnelled through pathway 1. However, if hydrolysis at the X - NH site precedes cleavage of Y, pathway 2 is followed. Product b' is "strongly resistant to further attack by hydrions because of the electrostatic shielding effect of $-NH_3^+$ group"⁴² so that under ordinary conditions of acid-catalyzed hydrolysis, the major portion of the products, because of slow cleavage, consists of resistant disaccharides.

Stacey⁴², in a study of model compounds, showed that pathway 2 was

followed to an appreciable extent (ranging between 14 - 37%). Some of these results are summarized in Table 4.

Compound	X	Y	Time of half hydrolysis (min.)	Glucosamine release (%)
2-Acetamido-2-deoxy-D-glucose	Ac	H	4 - 6	100
Methyl 2-acetamido-2-deoxy- α -D-glucopyranoside	Ac	OMe	36	78
Methyl 2-acetamido-2-deoxy- β -D-glucopyranoside	Ac	OMe	4 - 8	82
Methyl 2-amino-2-deoxy- α -D-glucopyranoside hydrochloride	H,HCl	OMe	8.5×10^3	2
Methyl 2-amino-2-deoxy- β -D-glucopyranoside hydrochloride	H,HCl	OMe	2.8×10^3	6

Table 4. Acidic Hydrolysis of some D-glucosamine derivatives (Hydrolysis performed in 1.16 N - HCl)⁴²

The influence of -NH_3^{\oplus} group is reflected in the large times of half hydrolysis of some of the model compounds.

The reactions in Fig. 8 operate during acidic hydrolysis and will prevent complete liberation of the aminosugar and thus lead to low values for the hexosamine content. In terms of the above discussion, the reasons for the low values of hexosamine determination in Extracts I & II are readily understood.

Quantitative Determination of Acetyl Group

The low hexosamine values frequently reported for polysaccharides are attributable to incomplete hydrolysis or destruction of the amino-

sugar or both. Since conditions which would result in maximum liberation of the aminosugar also have some destructive effect,⁴³ it was decided to determine the exact hexosamine content of the polysaccharides by an indirect method.

Amongst the methods available for quantitative determination of the acetyl group in N-acetyl compounds, the Freudenberg acid transesterification method⁴⁴ was the best. Trial experiments with N-acetylglucosamine of known purity using this method gave, as reported by others,⁵³ inaccurate results. Quantitative infrared analysis could have provided the answer, but due to lack of facilities other methods were resorted to.

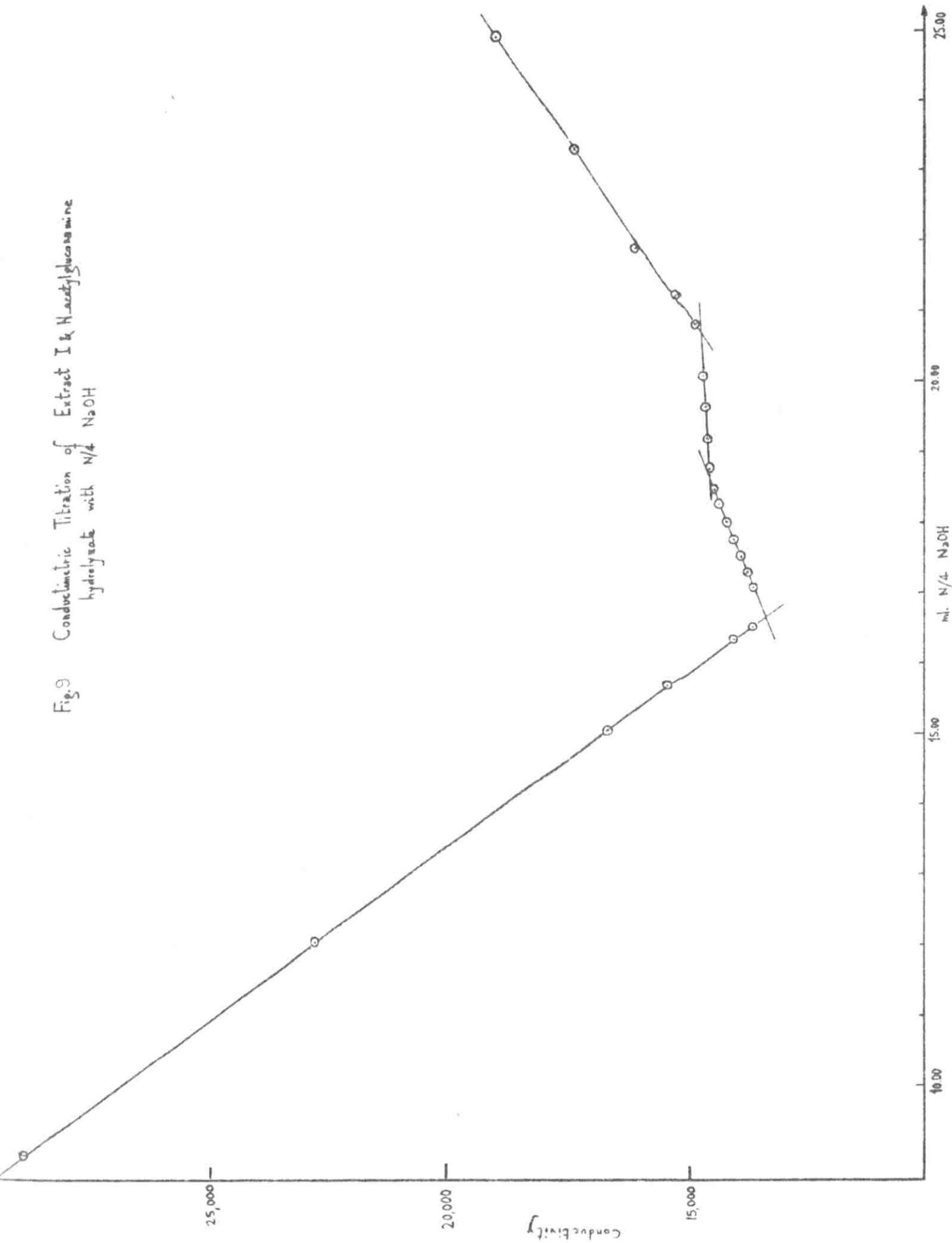
Potentiometric titration of a sample of the acidic hydrolyzate of the same compound did not lead to meaningful titration curves mainly due to lack of response of the glass electrode to sufficiently high pH values.

The novel application of conductimetric titration of the 1.16 N - HCl hydrolyzate of a mixture of Extract I and N-acetylglucosamine with $N/4$ NaOH gave good results. The method consisted essentially of the titration of a strong base with a mixture of a strong acid (excess HCl left from hydrolysis), a weak acid (HAc produced on cleavage of the acetamido group) and an ammonium salt (resulting after scission of the acetamido group). A typical titration curve is shown in Fig. 9. The acetyl content was determined from the titration curve and was found to be 12.5% (theoretical value 11.2%).

The acetyl value corresponded to 55.6% hexosamine content.

On the basis of the quantitative determinations, it can be stated that the major polysaccharide material of the hydatid cyst wall consists of equimolar amounts of galactose, hexosamine and acetyl group.

Fig. 9 Conductometric Titration of Extract I & H-acetylglucosamine hydrolyzate with N/4 NaOH



Optical Rotation

The specific rotations of polysaccharide Extracts I & II, determined in M - NaCl, were found to be $+4.2^{\circ}$ and $+38^{\circ}$ respectively. Since both polysaccharides were found to be structurally identical, this discrepancy could only mean that the anomeric linkages were of the β -type. On account of the alkali lability of the polysaccharide, Extract II was partially degraded, and hence had a higher specific rotation. Upon standing in solution, the rotation values of both polysaccharides were observed to increase. These findings, together with the results of the infrared analysis, provide a strong evidence for β -glycosidic linkages.

Identity of the Aminosugar

Due to the fact that glucosamine and galactosamine exhibit the same mobility on paper chromatography, and since other identification methods require relatively large amounts of material, the identity of the hexosamine was established by cation exchange chromatography on Dowex 50 W (H^{+} form). Gardell⁴⁵ showed that glucosamine and galactosamine could be separated quantitatively by ion-exchange chromatography on Dowex 50 (H^{+} form) resin column.

A column of Dowex 50 W X8 cation exchanger was calibrated with known amounts of authentic D-glucosamine hydrochloride and D-galactosamine hydrochloride. It gave sharp and quantitative separation of both aminosugars (Fig. 10). To an aliquot of the acid hydrolyzate of polysaccharide Extract I, a known amount of D-glucosamine hydrochloride was added, and the mixture was applied to the column. This time the

O.D. values refer to Elson-Morgan reaction (abs. max. 530 m μ)

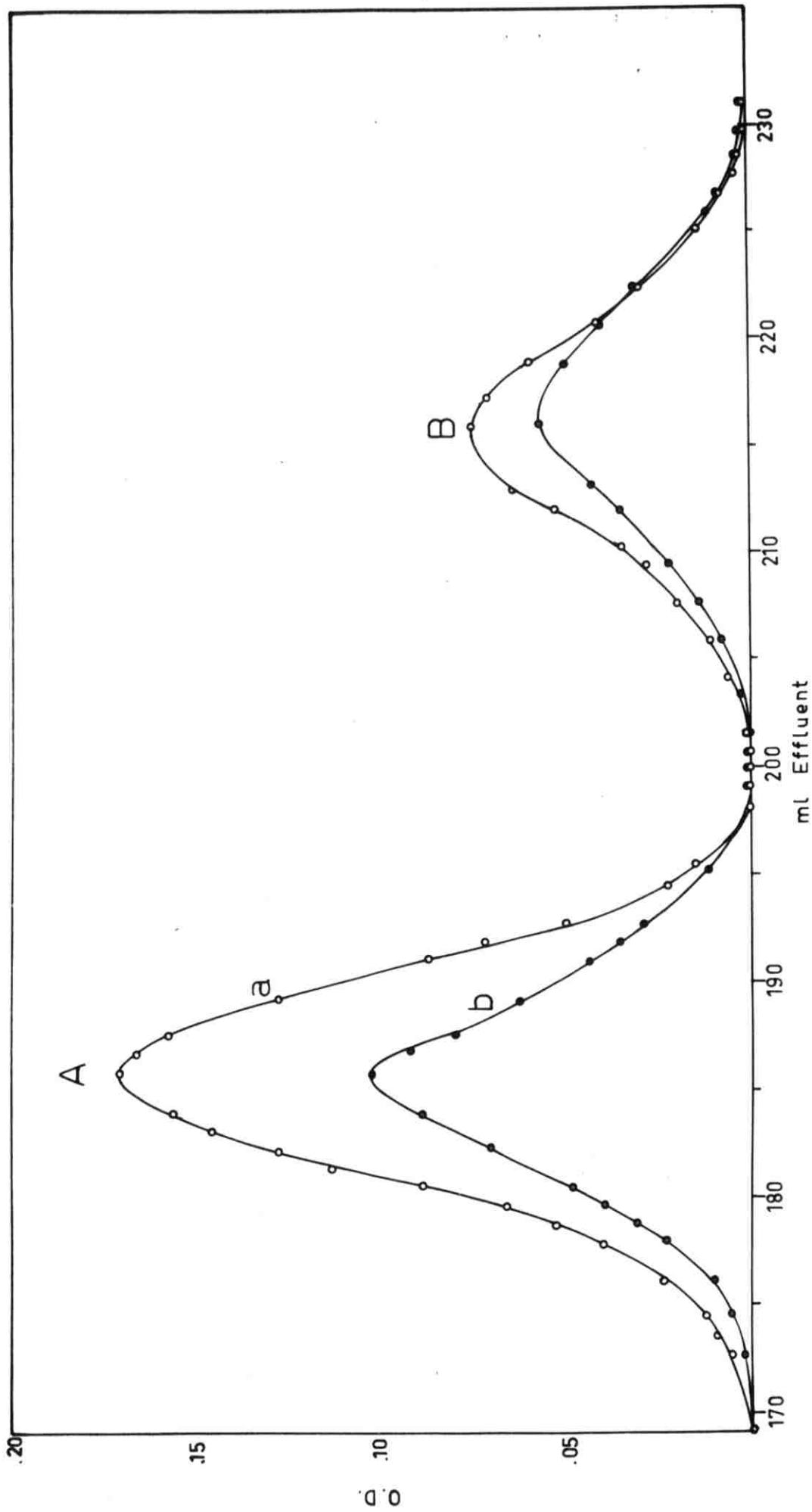


Fig. 40. Quantitative separation of glucosamine (A) from galactosamine (B) on a Dowex 50WX8 (200-400 mesh) column

- (a) Calibration of column with glucosamine - galactosamine mixture
- (b) Elution profile of glucosamine - polysaccharide (NaCl extract) hydrolyzate mixture

amount of glucosamine hydrochloride recovered corresponded to 120% of the authentic amount originally applied to the column. Thus, both aminosugars were present, but galactosamine constituted the major fraction. From the elution profile the ratio of galactosamine to glucosamine was shown to be 4:1. This finding disproves the results of all previous workers who, on the basis of paper chromatographic analysis alone, reported the identity of the hexosamine as glucosamine.

In the light of these results and the electrophoretic homogeneity of the polysaccharide two alternative suggestions concerning the distribution of the aminosugars can be advanced:

a) Both aminosugars may be incorporated in the same polysaccharide chain, a possibility arising from the likelihood that the enzymes of the organism may not be able to "differentiate" between both aminosugars during biosynthesis of the polysaccharide;

b) Each of the aminosugars may be a part of different chains; electrophoresis would then be considered as unable to separate any such two different polysaccharides.

Enzymatic Hydrolysis

Testicular hyaluronidase is classified as an endo-hexosaminidase because it attacks a glucosaminidic bond which is at least four monosaccharide units away from either end of the polysaccharide chain. The enzyme combines both hydrolytic and transglycosyl activities. Its hydrolytic action involves scission of only C₁ - O bond in the glucosaminidic linkages.⁴⁶ Upon prolonged digestion of hyaluronic acid with

the enzyme, the major hydrolysis products were identified as a tetrasaccharide and a hexasaccharide.

The hyaluronidase of bee venom was recently discovered by Barker and coworkers⁴⁷ to be a β -endo-hexosaminidase. It was shown to possess no transglycosyl action and did not cause any structural alterations due to elimination processes.

Samples of polysaccharide Extract I were incubated with bee venom hyaluronidase and testicular hyaluronidase for 120 hours. Hydrolysis products were examined by paper chromatographic analysis. The hydrolyzate of bee venom hyaluronidase gave a series of six relatively intense spots which possessed R_G values of 0.25, 0.45, 0.60, 1.02 (major oligosaccharide), 1.15 and 1.45 respectively. Testicular hyaluronidase on the other hand gave a hydrolyzate that consisted of two major degradation products with R_G values 0.25 and 0.50, and two minor degradation products with R_G values less than 0.25.

The action of both enzymes on Extract I proved that the hexosaminidic linkage was of the β -anomeric type and thus supported previous findings.

Suggestions for Future Work

Because of the considerable difficulties encountered in controlling the acid-catalyzed degradation of polysaccharides to any desired oligosaccharide stage, and in view of the susceptibility of the major polysaccharide constituent to the action of bee venom and testicular hyaluronidases, enzymatic degradation followed by examination of the degradation products would be of great help in further structural

investigations. Methylation and periodate oxidation studies applied to the fractionated degradation products may then lead to invaluable information on modes of linkages, ring size and branching of the polysaccharide chains.

EXPERIMENTAL

Source of Material

Echinococcus granulosus cysts obtained from infected sheep and cattle were emptied of cyst fluid by aspiration with a syringe. They were cut open, washed several times with tap water, and stored at -25° until extracted.

Isolation of the Polysaccharide Constituent

All operations, unless otherwise specified, were conducted in the cold at 4° .

Approximately 500 g. of frozen cyst membranes were allowed to thaw, and then were homogenized with acetone (250 ml.) in a Waring blender for 10 minutes. The resulting suspension was centrifuged in a MSE refrigerated centrifuge at 2500 r.p.m. for 12 minutes. The acetone extraction was repeated two more times. The residue (residue A) was then extracted, in the same manner, with three 200 ml. portions of chloroform-methanol (1:3 v/v). The resultant lipid-free tissue was freeze-dried in a VIRTIS lyophilizer and completely dried over P_2O_5 and NaOH pellets in a vacuum desiccator. The dry defatted material (residue B: 50.3052 g.) was then extracted with 2 M - NaCl (20 ml./g.) for 12 hours. Extraction was carried out in a Warburg apparatus, using tightly-stoppered conical flasks each containing 3 ml. of toluene to prevent bacterial growth. After centrifugation of the saline extract,

the tissue residue was dialyzed against running tap water for 72 hours, lyophilized and stored in the cold. (residue C: 29.80 g.).

The combined saline extracts were deproteinized at room temperature by repeated shakings (22x) with 30 ml. portions of Sevag's reagent (chloroform-amyl alcohol, 9:1 v/v) until no more gel-like material separated at the interphase. The protein-free saline extract was then dialyzed against tap water for 48 hours and lyophilized. The fluffy white material obtained (0.4650 g.) was designated as Extract I.

Residue C was extracted with N - NaOH (600 ml.) for 24 hours at room temperature with continuous mechanical stirring. The NaOH extract (Extract B) was neutralized with acetic acid to the end-point of phenolphthalein indicator, and dialyzed for 72 hours against tap water. The dialyzed solution was then concentrated by freeze-drying, rendered 2 M with NaCl and deproteinized by repeated shakings with Sevag's reagent. The protein-free saline solution was dialyzed against tap water for 48 hours and freeze-dried to give 0.3227 g. of white fluffy residue denoted as Extract II.

Both polysaccharide fractions were stored in the dry state in the deep-freeze.

The extraction procedure is schematically outlined in Fig. 1.

Extraction of Glycogen

Cystic membranes were extracted successively, as in the previous procedure, with acetone (3x) and chloroform-methanol (1:3 v/v)(3x). 20.0 g. of the dry defatted product (Residue B¹) was extracted with 30% KOH (400 ml.) for 1 hour at 100⁰, according to Pflüger's method. The

extract was neutralized with acetic acid to phenolphthalein end-point, dialyzed for 72 hours against running tap water and lyophilized to recover 3.0459 g. of brownish material (Extract C). This product was dissolved in 200 ml. of phosphate buffer (pH 7.20) and rendered 1 M with NaCl. The buffered saline solution was then deproteinized by full-speed agitation with buffer-saturated 1,2,2-trichloro-1,1,2-trifluoroethane^{48a,b} in a Waring blender and then centrifuged at 2000 r.p.m. in a refrigerated centrifuge at 4°. At this point three layers could be seen:

- a) an upper aqueous layer
- b) a middle gel-like layer, and
- c) a bottom layer of deproteinizing agent.

The upper water layer was removed and treated as above for nine more successive extractions. During such treatment further gel-like material was removed. The extract was further deproteinized by shaking (10x) with 100 ml. portions of Sevag's reagent, as in the original procedure. The protein-free aqueous phase was then dialyzed for 60 hours and freeze-dried to give 0.0108 g. light brown material designated as Extract III.

The extraction procedure is presented in Fig. 2.

Ultraviolet Analysis

A 1.0 mg. (+ 0.1) sample of each of Extracts I & II, dried in vacuo over P₂O₅ at 60°, was dissolved in 5.00 ml. of deionized distilled water. The solutions, in turn, were transferred into quartz cells of 1.00 cm. pathlength, and the ultraviolet spectra (200 - 420 mμ) were determined in

a Unicam spectrophotometer Model SP500, using the solvent as blank.

Infrared Analysis

A dried sample (3.0 ± 0.1 mg.) of each of Extracts I & II was dissolved in 25 ml. of deionized distilled water together with 350 mg. of potassium bromide (AnalaR). The solutions were then well stirred, frozen and lyophilized. The mixtures, ready for pressing into discs, were sent for analysis to the Chemistry Department of the University of Birmingham. The infrared spectra were recorded on a Perkin Elmer infrared spectrophotometer Model 21.

Hydrolyses

10 mg. of each polysaccharide material (Extracts I & II) was hydrolyzed with 0.5 ml. of 2 N - H_2SO_4 at 100° , for 4 hours. 4 mg. of Extract III was similarly treated with 0.2 ml. of the acid. Hydrolyses were conducted in small pyrex tubes fitted with air condensers. Hydrolyzates were then transferred to centrifuge tubes, neutralized with $BaCO_3$ to pH 7, centrifuged and the clear supernatants lyophilized.

Paper Chromatography

Approximately 1% solutions of all hydrolyzates were prepared. Glucuronic acid (potassium salt), galactose, glucosamine (hydrochloride salt) and glucose (all B.D.H. biochemicals) were used as reference standards. All paper chromatographic analysis was performed on Whatman No. 1 paper, using the descending technique.

Extracts I & II: Chromatograms were developed for 72 hours with the organic phase of n-butanol - acetic acid - water (4:1:5 v/v).

Extract III: Chromatograms were developed using the following solvent systems:

- a) Ethyl acetate - pyridine - water (2:1:2 v/v) - 22 hours
- b) n-butanol - pyridine - water (9:5:8 v/v) - 60 hours.

After irrigation, the chromatograms were dried, and the saccharide spots detected with one of the following spray reagents:

- a) aniline phthalate for reducing sugars,⁴⁹
- b) 0.05% ninhydrin (AnalaR) in ethanol⁵⁰ for certain aminosugars,
- c) silver nitrate,⁵¹ the dry chromatograms were first passed

rapidly through an acetone solution of AgNO_3 , and allowed to dry in air. Spraying with 0.5 N ethanolic NaOH brought up reducing and non-reducing sugars as black spots. When required as permanent records, the chromatograms were fixed by soaking them in 40% $\text{Na}_2\text{S}_2\text{O}_3$ solution followed by thorough rinsing with water.

Paper Ionophoresis

Paper ionophoretic analysis was performed by using strips (3 x 30 cm) of Whatman No. 3 ~~MM~~ paper in a Spinco-Durrum electrophoresis apparatus. Boric acid-borate buffer (pH 9.20) was used.

8.0 mg. of polysaccharide (Extract I) was dissolved in M - NaCl (4 ml.) and a total of 100 - 150 μl of this solution was applied. Ionophoresis was conducted at 450 v. for $4\frac{1}{2}$ hours and the paper ionophoretograms were sprayed with phenol red spray reagent.⁵²

Optical Rotation

Polysaccharide Extracts I, II & III were dried in vacuo over P_2O_5 for 4 hours at 60° , and adequate amounts were dissolved in M - NaCl solution. Determinations of optical rotation were made in a Lippich Polarimeter (Schmidt U. Haensch) with the direct-vision-spectroscope type monochromator using 1 dm. cells of 0.1 cm diameter.

Determination of Uronic Acid³⁵

A. Reaction in 87% sulfuric acid:

0.05% and 0.02% solutions of Extracts I & II in water were prepared. To 1 ml. of each solution, 6 ml. 87% sulfuric acid (diluted from 98% H_2SO_4 , B.D.H. Microanalytical Reagent) was added with cooling in ice-water. The reaction mixture was then heated for 20 minutes in a boiling water bath and subsequently cooled in tap water. 0.2 ml. of a 0.1% ethanolic solution of carbazole (commercial grade recrystallized twice from benzene) was then added and the solutions allowed to stand for 2 hours at room temperature. Optical density values of the colors developed were read at 535 $m\mu$ in a Hilger-Watts spectrophotometer against a blank. The reaction was also run on 1-ml. samples of a 0.02% solution of hyaluronic acid (Fluka AG, potassium salt) and a 0.05% solution of rabbit glycogen (Mann Research Lab.) for comparison.

B. Reaction in 98% sulfuric acid

The same reaction as above was conducted using 98% sulfuric acid instead of the 87%.

Morgan-Elson Reaction³²

To two 1-ml. portions of a 0.183% solution of Extract I in M - NaCl 0.10 ml. of 0.5 N - Na_2CO_3 was added. The resulting solution, after thorough mixing, was heated for 20 minutes in a vigorously boiling water bath and then quickly cooled to 0°. Glacial acetic acid (AnalaR) was then run into each tube to yield a total volume of 7 ml., and 1 ml. N,N-dimethyl-p-aminobenzaldehyde (DMAB) reagent (2% DMAB (AnalaR) in glacial acetic acid containing 2.5% (v/v) 10 N - HCl) was then added. The volume of liquid in each tube was then made up to 10 ml. by the addition of glacial acetic acid, the tubes were thoroughly mixed and allowed to stand for 90 minutes in the dark at room temperature. The solutions were then filtered through a fine sintered-glass funnel to give a clear violet solution. The absorption spectrum was recorded on a Perkin-Elmer UV-VIS spectrophotometer (Model 202) using 4.0 cm. silica cells versus a blank.

The determination was repeated using boric acid-borate buffer (pH 8.80) instead of 0.5 N - Na_2CO_3 .

Quantitative Determination of the Aminosugar⁴⁰

1 - 2 mg. dried samples of Extracts I & II were hydrolyzed with 3 N - and 4 N - HCl (both diluted from B.D.H., Microanalytical Reagent, S.G. 1.18) at 100° and 105° for periods between 4 - 18 hours using 0.5 - 0.75 ml. acid/mg. of polysaccharide. Hydrolyzates were quantitatively transferred to 10 ml. volumetric flasks and diluted to the mark with washings of the hydrolysis tubes.

In a typical run, to 1 ml. of the acidic solution one drop of phenolphthalein indicator was added and the minimum quantity of N - NaOH was run

to give a full pink color. The alkaline solution was neutralized with dilute HCl (approximately 0.3 N) to the discharge of the indicator color and 1 ml. of a freshly prepared 2,4-pentanedione reagent was added (1 ml. of B.D.H. acetylacetone, b.p. 138 - 140^o, in 50 ml. of 0.5 N - Na₂CO₃). After washing down the walls of each tube with distilled water to give a final volume of 3 ml., the contents were well mixed by gentle shaking. The tubes were covered with long-necked (2 - 3 cm) condensers each containing 2 - 3 ml. water, heated in a vigorously boiling water bath for 20 minutes and cooled to room temperature. To each tube 5.0 ml. ethanol was added, followed by 1 ml. of Ehrlich's reagent (0.8 g. of pure DMAB in 30 ml. of ethanol and 30 ml. HCl, B.D.H. Microanalytical Reagent, S.G. 1.18), and the final volume was made up to 10 ml. with ethanol. The contents of the tubes were thoroughly but gently mixed and warmed for 10 minutes in a water bath at 65 - 70^o to accelerate liberation of CO₂. After cooling to room temperature, the contents of the tubes were again mixed, and the color intensity at 530 m μ was measured in a Hilger-Watts spectrophotometer using 1.0 cm. silica cells. The absorption spectrum of the color produced was also recorded using a Unicam spectrophotometer (Model SP800).

Standard tubes containing 10, 20, 30, 40, 50 and 60 μ g glucosamine were treated in the manner described above. The calibration curve is shown in Fig. 11.

Hexose Determination - Orcinol-sulfuric Acid Reaction³⁸

0.7 - 1.3 mg. dry samples of Extract I were hydrolyzed with 3-N - H₂SO₄ at 100^o for 2, 3, 4, 5 and 6 hours. Hydrolysis was performed in

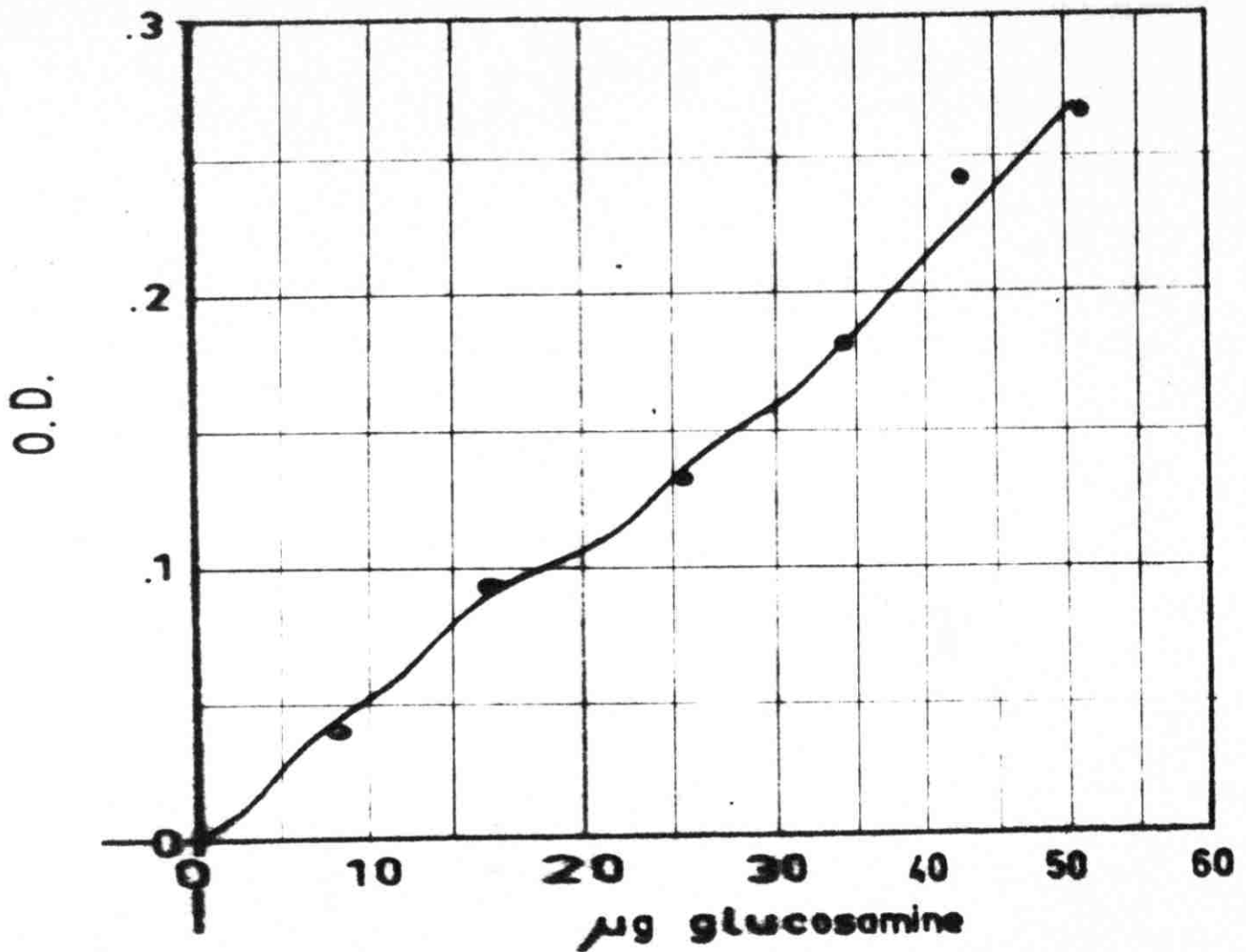


Fig. 11 ELSON - MORGAN REACTION
(Modif. of Rendle & Morgan)

Calibration curve for hexosamines

glass-stoppered tubes in a boiling water bath. Each of the hydrolyzates, after cooling, was transferred quantitatively to a 10 ml. volumetric flask and diluted to the mark with rinsings of the hydrolysis tube. Filtration was not necessary since the hydrolyzates were clear.

Three 2-ml. aliquots of each hydrolyzate were pipetted into test tubes. The rack containing the tubes was immersed in an ice bath for 15 minutes. 4 ml. of orcinol reagent (0.2 g. of orcinol (Merck) recrystallized twice from benzene, in 100 ml. 98% sulfuric acid) was then added dropwise to two of the samples, while 4 ml. of blank reagent (same sulfuric acid used for the reagent) was added to the third. During addition of the reagent and the blank, tubes were left immersed in the ice bath. After 15 minutes, the solutions were mixed thoroughly with stirrers (glass rods flattened at one end) and the rack was placed in a constant temperature bath at 80° for exactly 20 minutes. After heating the tubes were chilled in the ice bath and absorption readings were taken in a Hilger-Watts spectrophotometer at 505 m μ in 1 cm. cells.

Standards of 0, 25, 50, 75, 100 μ g galactose were treated under the same conditions. The amount of galactose was estimated from the calibration curve (Fig. 12) after subtraction of the sample blank reading.

Acetyl Determination

A mixture of polysaccharide I (16.2 mg.) and authentic N-acetylglucosamine (112.6 mg.) was hydrolyzed with 1.16 N - HCl (4.00 ml.) at 100° for 30 minutes in sealed tubes. The resultant hydrolyzates were then titrated conductimetrically with 0.25 N - NaOH using a conductivity bridge (Model RC 16B2, Industrial Instruments, Inc.).

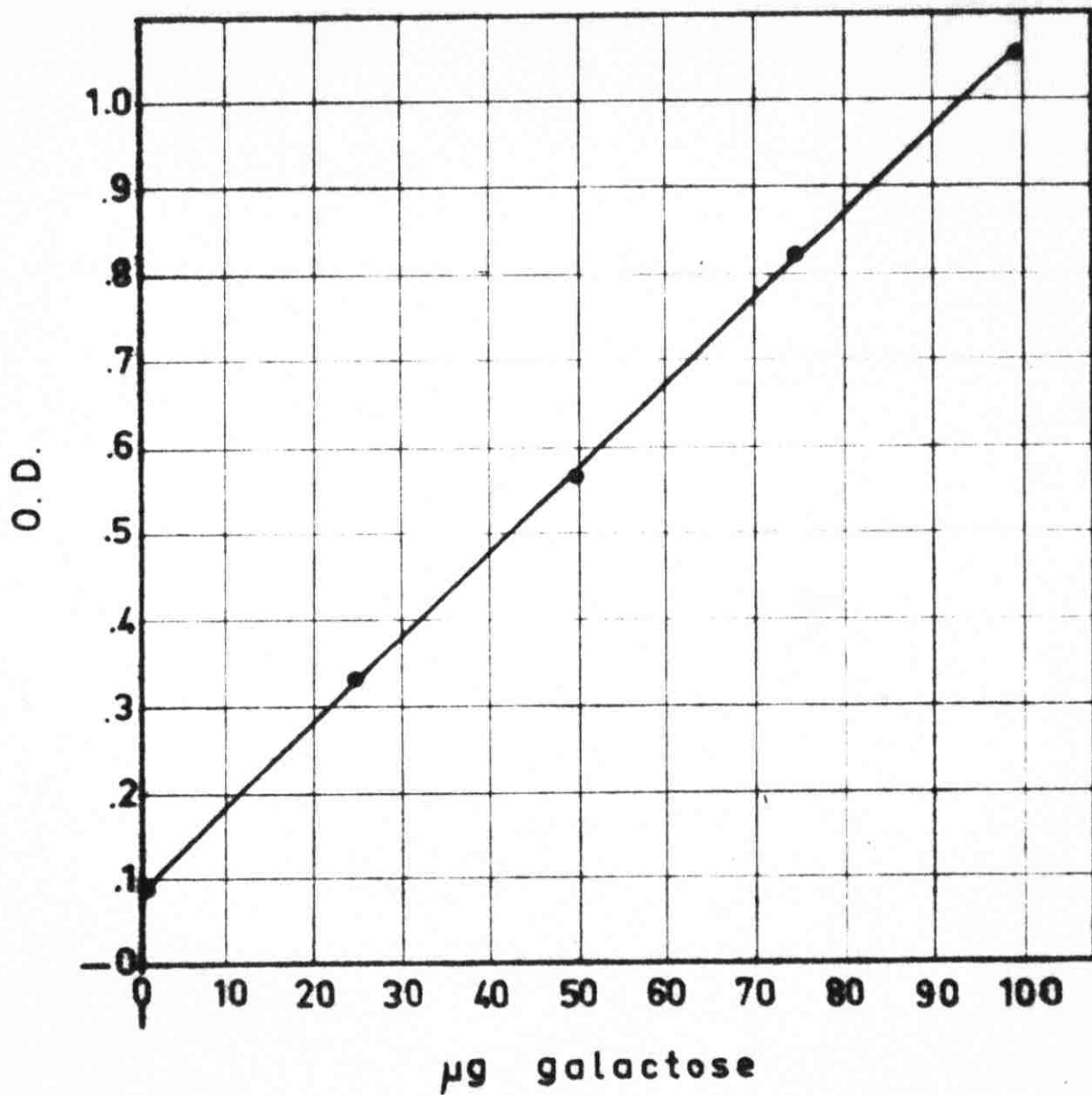


Fig. 12 ORCINOL - H₂SO₄ REACTION
Calibration curve for hexoses

The conductivity values were plotted as a function of volume of base added (Fig. 9). The acetyl value was calculated, from the equivalents of acetic acid as determined from the plot, after subtraction of the blank of same aminosugar content.

Identification of the Aminosugar by Cation Exchange Chromatography⁴⁵

A. Preparation of Column:

42.00 g. of Dowex 50 W X8 (200/400 mesh) was washed with a total of 85 ml. portions of 4 N - HCl to rid the resin of fine particles. The resin was then suspended in 4 N - HCl and poured all at once into a buret (45 x 1.1 cm.) two thirds full of 4 N - HCl and packed with a piece of pyrex glass wool at the bottom. The resin was left to settle overnight, and the column was washed with 0.3 N - HCl for 26 hours, after which time the normalities of inflowing and outflowing solutions were identical.

B. Application of the Material:

0.3 ml. of a 0.3 N - HCl solution containing authentic glucosamine hydrochloride (233 µg.) and the lyophilized hydrolyzate resulting from 2 mg. of Extract I was applied to the column. The solution was allowed to run slowly through and the walls were washed twice with 0.5 ml. portions of 0.3 N - HCl. The washings were also allowed to pass through slowly. The space above the column was then filled with 0.3 N - HCl and the column was connected to a 0.3 N - HCl reservoir. The level of the latter was adjusted to give a flow rate of 1 - 2 ml. effluent/hour.

1-ml. fractions were collected on an automatic fraction collector (Gallenkamp) starting from the moment the mixture was applied to the column. For the maintenance of the same flow rate the level of the reservoir was occasionally altered.

C. Calibration of the Column:

0.65 ml. of a 0.3 N - HCl solution containing 502 μ g of glucosamine hydrochloride and 250 μ g of galactosamine hydrochloride was applied to the column. The column was eluted with 0.3 N - HCl at a flow rate of 1 - 2 ml./hour as above. Aminosugar content in the fractions was determined according to the Elson-Morgan technique.⁴⁰

Enzymatic Hydrolysis

A. Bee Venom Hyaluronidase:

4.0 mg. of Extract I in 0.24 ml. physiological saline (0.9% NaCl solution) was incubated with 2.0 mg. bee-venom hyaluronidase in 0.16 ml. phosphate-citrate buffer (pH 4.75) at 37^o for 5 days. At 24-hour intervals an additional 0.5 mg. of enzyme was added.

B. Testicular Hyaluronidase:

The above procedure was repeated using testicular hyaluronidase (B.D.H. biochemical, from ovine testes, 300 - 500 I.U./mg.) instead of the previous enzyme.

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