STRUCTURAL STUDIES

ON THE

POLYSACCHARIDE MATERIAL

OF

TAENIA HYDATIGENA CYST MEMBRANE

BY

DENISE C. CHAIBAN

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

June, 1966

STRUCTURAL STUDIES

ON THE

POLYSACCHARIDE MATERIAL

OF

TAENIA HYDATIGENA CYST MEMBRANE

BY

DENISE C. CHAIBAN

ACKNOWLEDGMENT

The author acknowledges with pleasure the encouragement and conscientious support of Professor Shibli I. Bayyuk who directed this investigation.

She also wishes to thank the Department of Tropical Health (AUB) for the supply of Taenia hydatigena cyst membranes, Mr. R. Guy for the determination of the infrared spectra and Mr. S. Ishak for providing all laboratory facilities.

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

ABSTRACT

The polysaccharide constituent of the cyst membrane of Taenia hydatigena was isolated, purified and its structure examined. It constituted approximately 5.7% of the dry defatted membrane tissue and consisted of only one component. 61.4% of this polysaccharide occurred firmly bound to proteins and required 1 M NaOH for extraction, while the rest occurred either in the free form or in loose combination with proteins..

The saline-extracted fraction of this polysaccharide had $[\alpha]_D$ + 133°, while the alkali-extracted fraction had $[\alpha]_D$ + 120°. Qualitative and quantitative analyses indicated that the polysaccharide consisted of only one monosaccharide building unit which was identified as glucose. The infrared spectrum of the polysaccharide was typical of that of glycogen. Optical rotation measurement and enzymatic studies with α - and β -amylases indicated that the D-glucose units were α -(1 --> 4)-linked in the chains. Thus structurally, the polysaccharide could be considered as a certain type of glycogen, which exhibited a much higher degree of branching than reference rabbit liver glycogen but had shorter exterior chains.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Purpose of the Work

Reliable information about the structure of the polysaccharide constituent(s) of Taenia hydatigena cyst membrane is grossly lacking. This is obviously attributable to the fact that very little attention has been paid to the structural identification of this material. The only attempt reported to reveal the chemical nature of this polysaccharide material was based on infrared spectroscopic study of fixed samples of the cyst wall.

The purpose of the present investigation was to isolate the polysaccharide material from the cystic membranes under the mildest possible conditions in a pure form, to examine its homogeneity, to fractionate if necessary so as to obtain the pure component fractions separately, and finally to attempt the characterization of each polysaccharide fraction by studying its chemical structure. This will increase our knowledge about the nature of the polysaccharides of cestodes in general, and secondly, will shed more light on the chemistry of the structural components and metabolic products of T. hydatigena in particular which is essential for immunological studies.

Life Cycle of the Parasite and Historical

T. hydatigena is a cestode parasite that completes its life cycle by infecting the intermediate host which is mainly sheep. Thus it

presents an economic problem in all sheep-raising countries. Unlike Echinococcus granulosus which infects man as an intermediate host, T. hydatigena does not.

The adult T. hydatigena tapeworm consists of a scolex and few segments (proglottids), and lives in the intestine of the definitive host which is the dog. When mature, the last proglottid becomes gravid, gets detached and excreted with the dog's feces. Upon exposure, the gravid proglottids release their ova, each of which contains an oncosphere which is a small spherical embryo with three pairs of claw-like hooks. If ingested by the intermediate host (sheep), an ovum hatches and the liberated larva makes its way through the intestinal wall into the blood stream which conducts it to the liver where it develops into an immature cysticercus,[1] (a bladder worm with one invaginated scolex). Such a cysticercus causes haemorrhagic streaks in the liver, and after a certain period of maturation, it pierces the liver tissue and moves to the abdominal cavity where it develops into a mature cysticercus. It is significant to note that the cysticerci do not become infective unless they develop hooks and suckers. Thus most hepatic cysticerci are not infective until they migrate to the surface of the liver or into the abdominal cavity of the intermediate host.[2] The life cycle of the parasite is completed when a mature cysticercus is ingested by the definitive host. In such an instance, the scolex survives, evaginates, attaches itself with the aid of the hooks and suckers to the mucous membrane of the intestine and grows into an adult worm.[1]

During the investigation of the host-parasite relationships in echinococcosis, Kilejian, Sauer and Schwabe[3] identified the polysaccharide constituent of the cyst wall of T. hydatigena as glycogen. They based their characterization on evidence obtained from infrared spectra of the dried cyst wall.

While infrared spectra[4-6] can ably assist in identification of the chemical structure of tissue polysaccharides, they cannot be used as the only criterion in establishing the structure of such complex constituents, especially if crude tissue material is used. Thus the lack of information substantiated by reliable experimental evidence prompted the initiation of the present work.

RESULTS AND DISCUSSION

In this study only membranes of Taenia hydatigena cysts obtained from infected sheep were used. The cysts were freed of their fluid after excising the scolices, and the membranes obtained were washed with water and stored at -25° until extracted. This ensured inhibition of degradation of any structural component by enzymatic activity prior to extraction.

Extraction

The purpose of the extraction was to isolate the polysaccharide constituent in[a] pure form that represented, as closely as possible, the original state in which it occurred in the membranes. As a principle, to effect minimum degradation of the polysaccharide component, neutral solutions should be used, and the extraction must be conducted at a low temperature. Acidic and basic solutions, drastic conditions and relatively high temperatures usually yield degraded products. Hence in this work, a very mild extraction procedure was adopted.

The membranes were homogenized and extracted at 4°, first with acetone and then with chloroform-methanol (1:3 v/v) to remove lipids, phospholipids, lipopolysaccharides and any colouring material. This step was essential, otherwise the lipids would interferewith the extraction process, and would be very difficult to get rid of at

a later stage. The polysaccharide constituent of the defatted dry tissue was isolated by extraction at 4° with 2 M NaCl saturated with toluene (to inhibit bacterial growth). The saline extract was deproteinized by repeated shakings with chloroform-amyl alcohol (9:1 v/v) according to Sevag's[14] method (Fig. 1). Upon dialysis and lyophilization 0.6725 g of polysaccharide material (2.2% of the dry defatted tissue) was obtained and designated Extract I. This fraction consisted of the polysaccharide component that occurred either in the free form or loosely bound to proteins.

Since most of the known tissue polysaccharides contains acidic functional groups (carboxylic acid and /or sulfate) and would be expected to occur in firm combination with proteins, the tissue residue left after saline extraction was subjected to drastic extraction with N NaOH at room temperature for 24 hours. After neutralization with glacial acetic acid, the extract was deproteinized by the Sevag method, dialyzed against running tap water and freeze-dried. The polysaccharide component obtained weighed 1.0665 g (3.5% of the dry defatted tissue), and was called Extract II (Fig. 1). This fraction represented the polysaccharide constituent that occurred firmly bound to proteins.

It is apparent that most of the polysaccharide material (61.4%) of the membranes occurred firmly bound to proteins, while a minor fraction (38.6%) occurred either free and / or loosly bound to proteins.

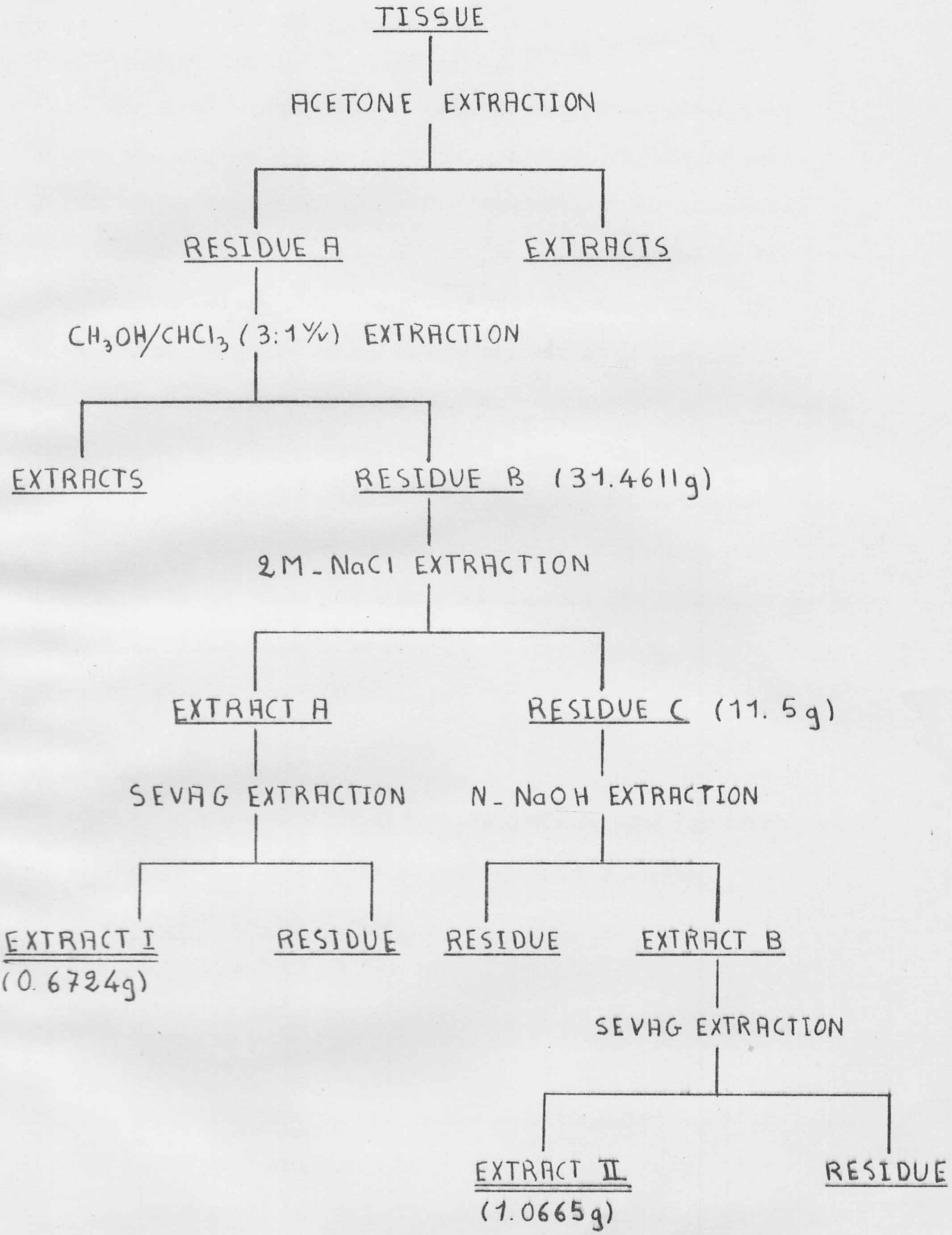


FIG. 1 : SCHEMATIC REPRESENTATION OF THE EXTRACTION PROCEDURE

Examination of the Purity of Extracts I and II.

The usual contaminants of polysaccharides extracted from animal tissues are nucleic acids and proteins. To detect any contamination with these impurities, the ultraviolet absorption spectrum of each polysaccharide fraction was determined in the region 200 - 420 mμ. The spectra of both fractions (Fig. 2) indicated the absences of any detectable amount of nucleic acids (λ_{max} around 260 mμ) and proteins (λ_{max} around 275 mμ). Elementary analysis by sodium fusion, however, gave a positive test for nitrogen. Since both polysaccharide fractions did not contain any aminosugar (will be shown later), the source of nitrogen was attributed to contamination with trace amounts of proteins or to the presence of small amounts of a firmly bound polysaccharide - protein complex.

Electrophoretic Mobility

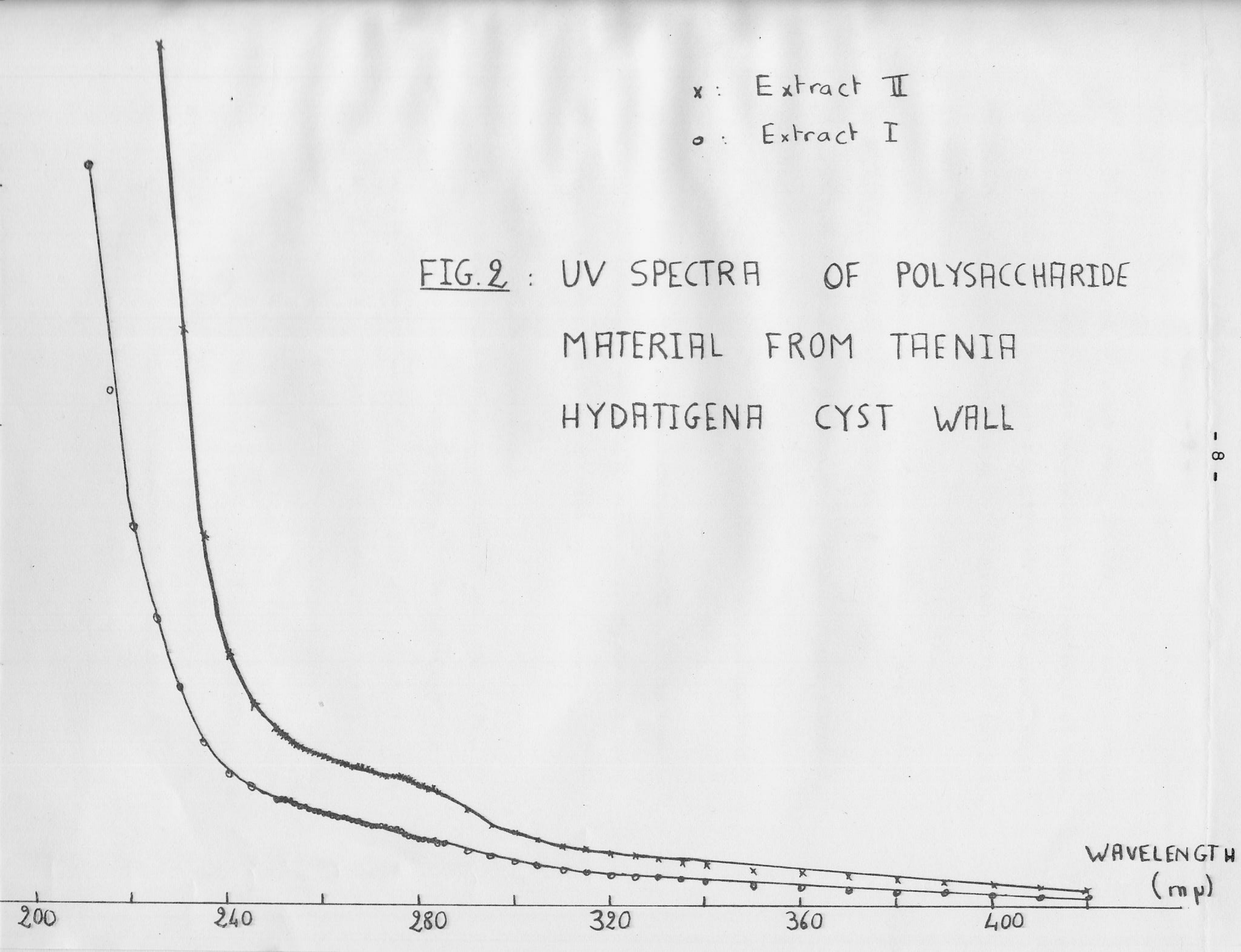
Several physico-chemical methods could be used for determination of the homogeneity of a polysaccharide. These include:

1. fractional solubility
2. diffusion
3. study of the sedimentation pattern in an analytical ultracentrifuge[15-17]
4. electrophoretic mobility of the polysaccharide using different buffers
5. serological activity in case the polysaccharide is biologically active.[18]

x : Extract II

o : Extract I

FIG. 2 : UV SPECTRA OF POLYSACCHARIDE MATERIAL FROM TAENIA HYDATIGENA CYST WALL



The first method is rather crude, and due to certain instrumental limitations, electrophoresis was chosen to give evidence of homogeneity. When the electrophoretic mobility of both polysaccharide fractions was examined on paper using 0.1 M borate buffer[19] (pH 9.2), it was found that:

a) each fraction gave two bands, one with 0.75 x the mobility of the other.

b) both bands of each polysaccharide fraction moved faster than rabbit glycogen.

c) the two bands of both polysaccharide fractions exhibited identical mobility.

This suggested that polysaccharides I and II could be very similar if not identical; and if both prove to be glycogen, it must be a certain type which is different from rabbit glycogen.

Infrared Spectrum of Polysaccharide I

The infrared absorption spectrum of polysaccharide I (Fig. 3) was determined in potassium bromide discs.[20] The polysaccharide exhibited strong absorption in the 3220 - 3360 cm^{-1} region attributable to O - H and N - H stretching, and weaker absorption at 2880 - 2910 cm^{-1} due to C - H stretching.[21] Absorption at 1410 - 1425 cm^{-1} could be ascribed to CH_2OH and CHOH groups.[22] Peaks in the region 1000 - 1220 cm^{-1} are mainly attributed to the C - O stretching modes and C - O - H bending modes,[23] and hence this region is of little significance for characterization purposes due to the abundant occurrence of ether and hydroxyl groups in polysaccharides.

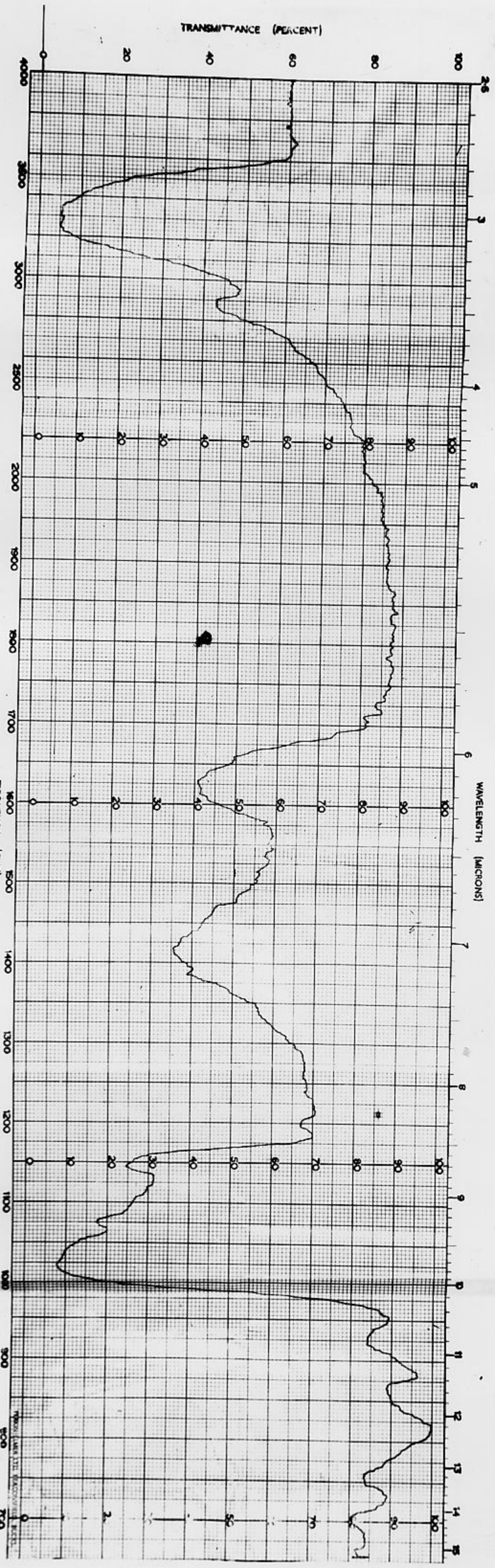


Fig. 3 - Infrared spectrum of Extract I

The most interesting part of the infrared spectrum was manifested in the fingerprint region (700 - 1000 cm^{-1}). The peaks at 925 - 930 cm^{-1}, 850 - 860 cm^{-1} and 750 - 765 cm^{-1} are characteristic of α - (1 $\longrightarrow$ 4) glycosidic linkages[24] and are typical of glycogen (peaks at 930 cm^{-1}, 835 $\pm$ 11 cm^{-1} and 765 cm^{-1}).

It can be concluded that the spectrum was typical of glycogen or a glycogen-like polysaccharide with α - (1 $\longrightarrow$ 4) glycosidic linkages. The other outstanding features were:

1) Absence of monosubstituted amides which, if present, would be readily detectable by their/characteristic bands at the 1645 cm^{-1} (C = O stretching vibration) and at 1560 cm^{-1} (N - H deformation).[24] No absorption was observed at 1375 cm^{-1} which also indicated absence of CH_3 groups associated with -NH - $\overset{O}{\overset{\|}{C}}$ - CH_3 groups. The peak at about 1620 - 1630 cm^{-1} is attributable to the presence of moisture, or -OH groups which are quite abundant in the polysaccharides.[4]

2) Absence of sulfate groups[24] (strong absorption at 1230 - 1250 cm^{-1}) and unionized carboxylic acid and lactone groups[24] (absorption at 1735 cm^{-1}), which indicated that the polysaccharide was neutral (no uronic or sulfuric acid groups). Further evidence for the absence of sulfate groups is provided by the elementary analysis which also indicated absence of sulfur.

3) Absence of β -glycosidic linkages[24] as evidenced from absence of absorption at 895 $\pm$ 9 cm^{-1} and 774 $\pm$ 9 cm^{-1}.

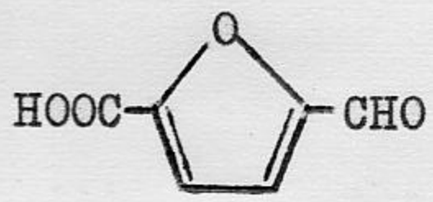
While tissue polysaccharides contain acidic mucopolysaccharides (sulfate and / or carboxylic group + hexosamines) in addition to the

neutral components, the polysaccharide constituent of the cyst membrane is quite unique because it consists of only what appears to be a certain type of glycogen.

Absence of Hexuronic Acids

The absence of uronic acids was established by the negative results obtained from Dische's carbazole colorimetric reaction.[13] According to this reaction, a uronic acid-containing polysaccharide gives a purple colour (absorption maximum at 535 mμ) when treated with concentrated sulfuric acid followed by an ethanolic solution of carbazole.[25]

The reaction is based on the formation of 5-carboxy-2-formyl-furan (I), which seems to be the chromogen responsible for the development of the colour in the presence of concentrated sulfuric acid.[26]



(I)

The absorption follows Beer's law in the range 5 - 100 γ hexuronic acid per ml and can be used for quantitative determinations.[25] Hexoses under the same conditions yield a brown-red colour with a completely different absorption spectrum.

The carbazole reaction was performed on authentic rabbit glycogen, polysaccharide I and authentic hyaluronic acid. A brown-red colour with an absorption maximum at 435 mμ was produced by rabbit glycogen

and polysaccharide I, whereas hyaluronic acid gave the characteristic purple colour that had an absorption maximum at 535 mμ.

The above results provide straightforward evidence for the complete absence of hexuronic acids in polysaccharide I, and hence support the previous infrared spectroscopic findings.

Preliminary Identification of the Monosaccharide Units

The monosaccharide building units of polysaccharides I and II were identified by paper chromatographic analysis of acid hydrolyzates (2N H_2SO_4 for 4 hours at 100^0), using the organic phase of the following systems as developers:

1) ethylacetate - pyridine - water (2:1:2 v/v)[27]

2) 1-butanol - pyridine - water (9:5:8 v/v)[28]

3) 1-butanol - acetic acid - water (4:1:5 v/v)[29,30]

Both polysaccharide fractions were found to be composed of the same monosaccharide residue - glucose only (Fig. 4). It was obvious that glucuronic acid and hexosamines were completely absent, and thus both polysaccharides could be described as polyglucosans. The positive nitrogen test in the elementary analysis, and the absence of hexosamine in both polysaccharide fractions can only be interpreted by the presence of trace amounts of polysaccharide - protein complexes and / or protein contaminants.

The glucose content of polysaccharides I and II was determined quantitatively according to Svennerholm's modification[11] of Vasseur's method.[31] The reaction is based on the fact that in the presence of concentrated H_2SO_4, hexoses are transformed into hydroxymethyl/formyl furan (II),

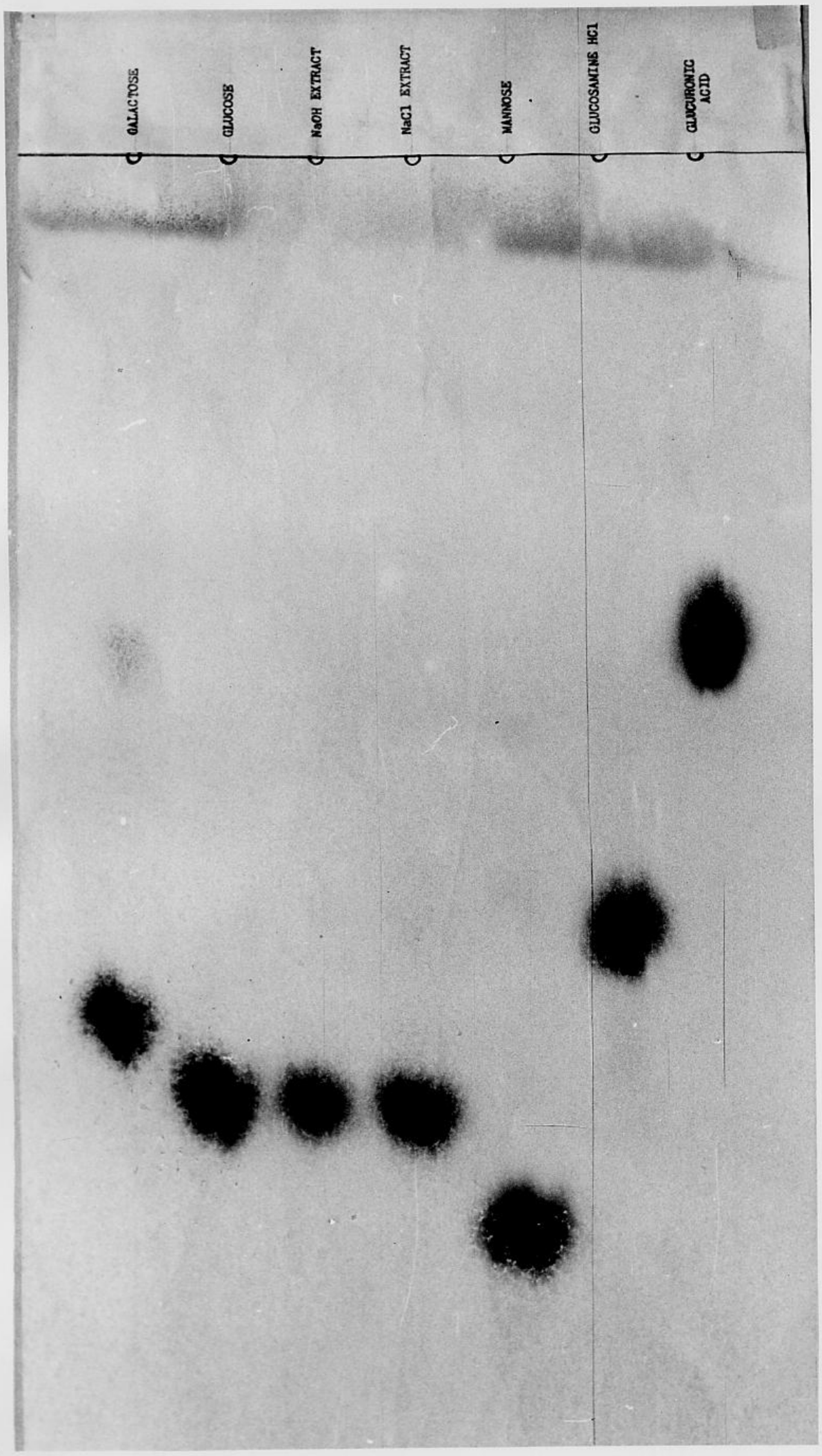
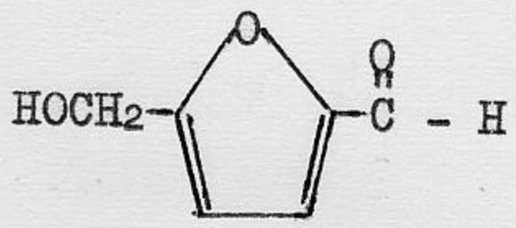


Fig. 4 - Paper Chromatogram of the Acid Hydrolyzates of Extracts I and II.

which condenses with phenolic compounds to give a dark red colour.



(II)

To determine the optimal time for the preliminary hydrolysis, polysaccharides I and II were treated with 3N H_2SO_4 for different periods ranging between 2 - 4 hours at 100°. The glucose content of each hydrolyzate was determined with the orcinol reagent (orcinol in 98% H_2SO_4). The optical densities of the samples were measured at 505 mμ against a blank and reference standards. The results obtained are tabulated in table I below. Protracted hydrolysis resulted in destruction of glucose.

The results of the quantitative determination indicated that glucose is the only monosaccharide building unit in both polysaccharides, and that polysaccharides I and II were closely similar if not identical.

Optical Rotation

The specific rotations of the polysaccharide fractions I and II were determined in M NaCl and found to be + 133°, and + 120° respectively. This provided further support for the presence of ∝ -anomeric linkages as revealed by infrared spectroscopy. Such high optical rotation values are characteristic of polysaccharides with ∝-glycosidic linkages. Polysaccharides with β -type linkages usually exhibit small negative rotations or no optical activity. Furthermore, since the polysaccharides I and II were found to be structurally identical, the discrepancy in their

TABLE I

ORCINOL-H_2SO_4 REACTION FOR D-GLUCOSE

Time of hydrolysis (hours)	% D-glucose	
	Extract I	Extract II
2	111	72.8
$2\frac{1}{2}$	---	96.5
3	---	84.6
$3\frac{1}{2}$	---	60.6
4	---	42.2

specific rotations lends further evidence for ∝ -glycosidic linkages. Since alkali-extraction yields partially degraded material, therefore a drop in specific rotation is characteristic of ∝ -linked polysaccharides.

Interaction with Iodine

Iodine is known to form inclusion complexes with polysaccharides in which the iodine molecules become arranged inside a series of helices of ∝ -(1 --→ 4)-linked D-glucose units. It was found that each helix of 6 D-glucose units complexes with one iodine molecule.[32,33] With branched ∝ -D-(1 --→ 4)-glucans, complex formation is probably limited to the exterior chains. The iodine-binding power of branched ∝ -D- (1 --→ 4)-glucans seems to be related to the exterior chain length. It was also observed that the optical density at the absorption maximum increases as the chain length increases. As to the absorption maximum of the iodine complexes of branched ∝ -D-(1 --→ 4)-glucans, it seems to be directly related to the exterior chain length.[34]

In order to assess the relative chain length of polysaccharides I and II, the absorption maxima of their iodine complexes and that of the iodine complex of reference rabbit liver glycogen were determined. The iodine complexes of polysaccharides I and II exhibited absorption maxima at 435 mμ, while that of rabbit glycogen at 520 mμ. Thus it is apparent that the exterior chain lengths in rabbit glycogen are longer than those of polysaccharides I and II, and the exterior chains of both polysaccharides are approximately of equal length. Furthermore the UV spectra of the iodine complex (Fig. 5) in 0.2% iodine solution

o: Extract II

Δ : Extract I

x : glycogen (rabbit liver)

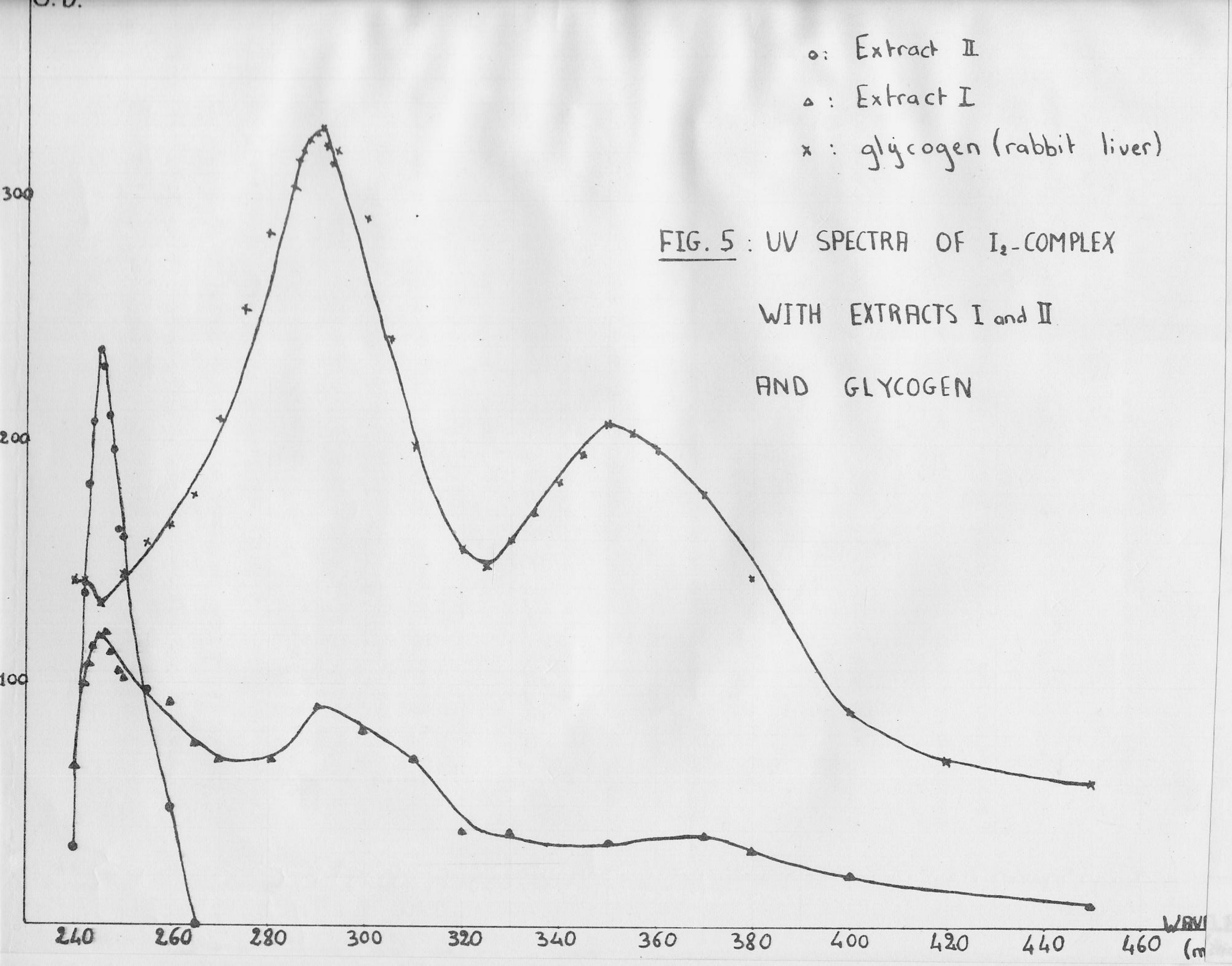


FIG. 5 : UV SPECTRA OF I_2-COMPLEX WITH EXTRACTS I and II AND GLYCOGEN

was recorded. Polysaccharides I and II absorbed at a shorter wavelength than reference rabbit glycogen.

Enzymatic Hydrolysis

β-amylase catalyzes the stepwise hydrolysis of polysaccharides consisting of α-(1 --> 4)-linked D-glucose units. Its action commences at the non-reducing end of a chain, and continues as long as the linkages are of the α-D-(1 --> 4) type. Amylolysis with this enzyme stops at glycosidic linkages other than α-D-(1 --> 4). Therefore β-amylase begins acting on the exterior chains, clipping off maltose units, and stops at the branching points.[36] α-amylase, on the other hand, catalyzes random hydrolysis of α-D-(1 --> 4)- glucose chains. It acts both at the exterior ends as well as on the interior of such chains. The immediate amylolytic products consist of maltose units and higher saccharides, but upon prolonged action, maltose is produced as the end product. The action of α-amylase stops at glucosidic linkages α-D-(1 --> 6).[36]

To assess the relative length of the exterior branches of the polysaccharide of T. hydatigena cyst membrane, polysaccharide I and reference rabbit liver glycogen were incubated with β-amylase. The enzymatic hydrolyzates were examined after 2,4,6,8,10 and 24 hours by paper chromatographic analysis using maltose as a reference standard. The chromatograms were irrigated with n-butanol: acetic acid: water (4:1:5 v/v). The results (Fig. 6a and 6b) indicated that polysaccharide I had shorter exterior chains than rabbit glycogen as evidenced from the intensity of the spots.

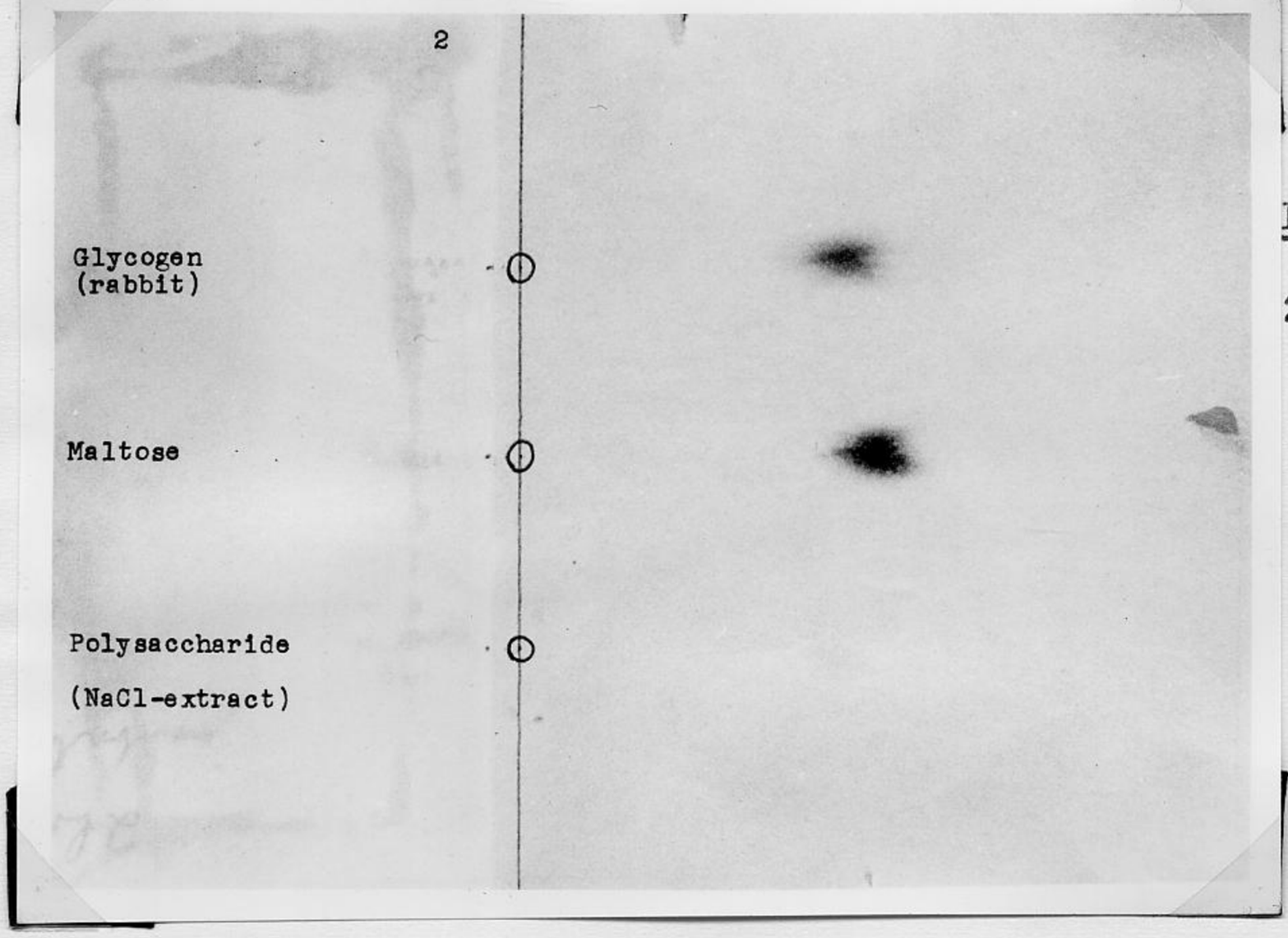


Fig. 6a

2 hours

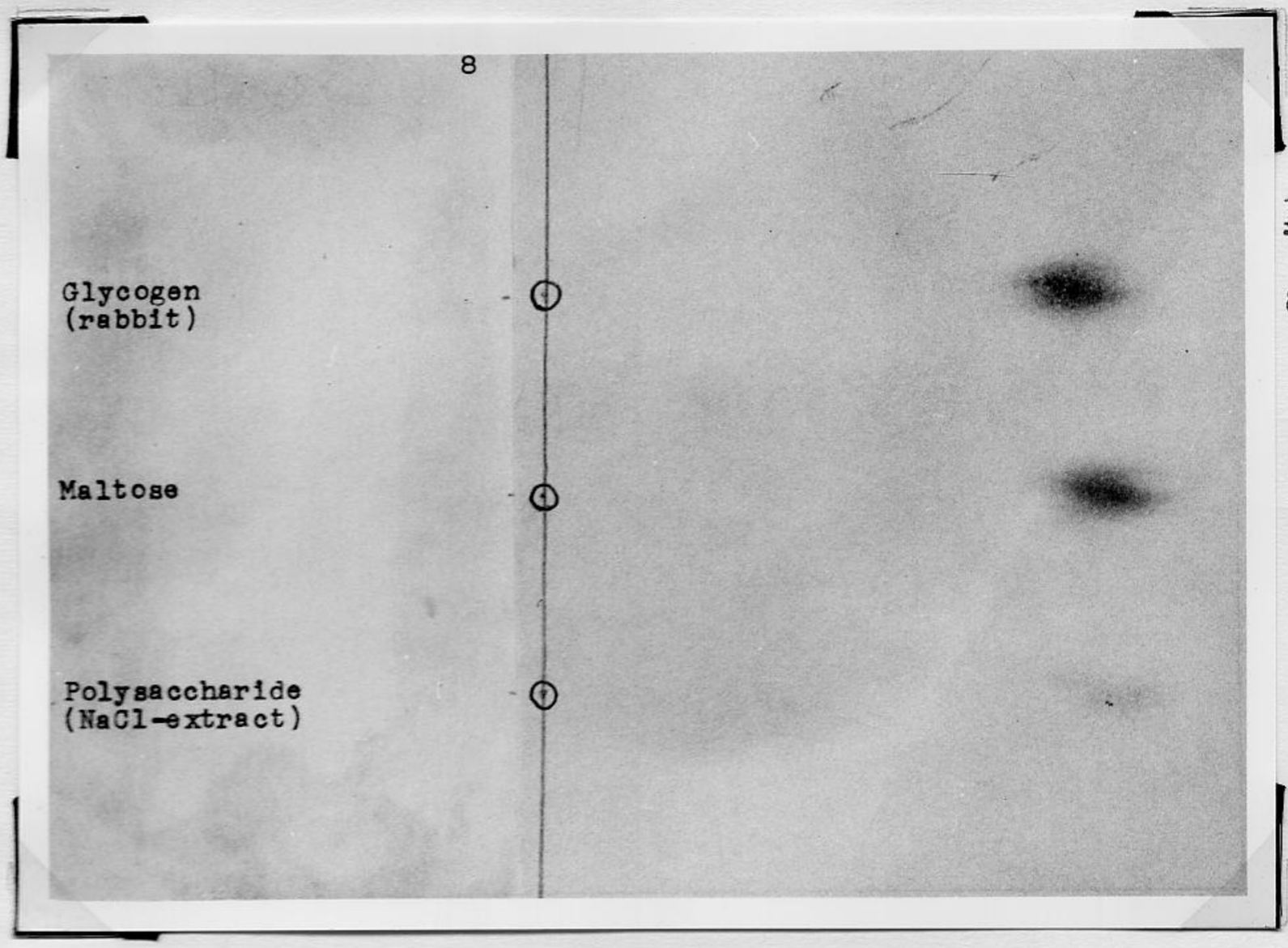


Fig. 6b

8 hours

Fig. 6 - Paper Chromatograms of Amylolysis on Polysaccharide I with β-amylase.

The relative degree of branching of polysaccharide I was assessed by treatment with ∝-amylase using rabbit liver glycogen as reference standard. It could be seen from Fig. 7a, 7b and 7c that the polysaccharide exhibited a higher degree of branching. To sum up, amylolysis of the polysaccharide with ∝- and β-amylases indicated that it was a branched glucan containing D-glucose units that were ∝-(1 ⟶ 4)-linked. All evidence points that structurally, it can be considered as a certain type of glycogen which is highly branched and with relatively short exterior chains.

To estimate the relative degree of branching and the relative length of the exterior chains, polysaccharide II and reference rabbit glycogen were incubated with ∝- and β-amylases. At different intervals aliquots of both hydrolyzates were analyzed for reducing sugars, using Nelson's modification[35] of Somogyi's method. The results obtained are shown in Table II.

Thus polysaccharide II had exterior chains approximately half the length of those of rabbit glycogen, and exhibited almost double the degree of branching of the reference glycogen.

In conclusion, it can be deduced that the polysaccharide constituent of T. hydatigena cyst membrane consisted of only one component. This component proved to be a glucan containing ∝-(1 ⟶ 4)-linked D-glucose units. Structurally it could be considered as a new type of glycogen which exhibited a high degree of branching with short exterior chains.

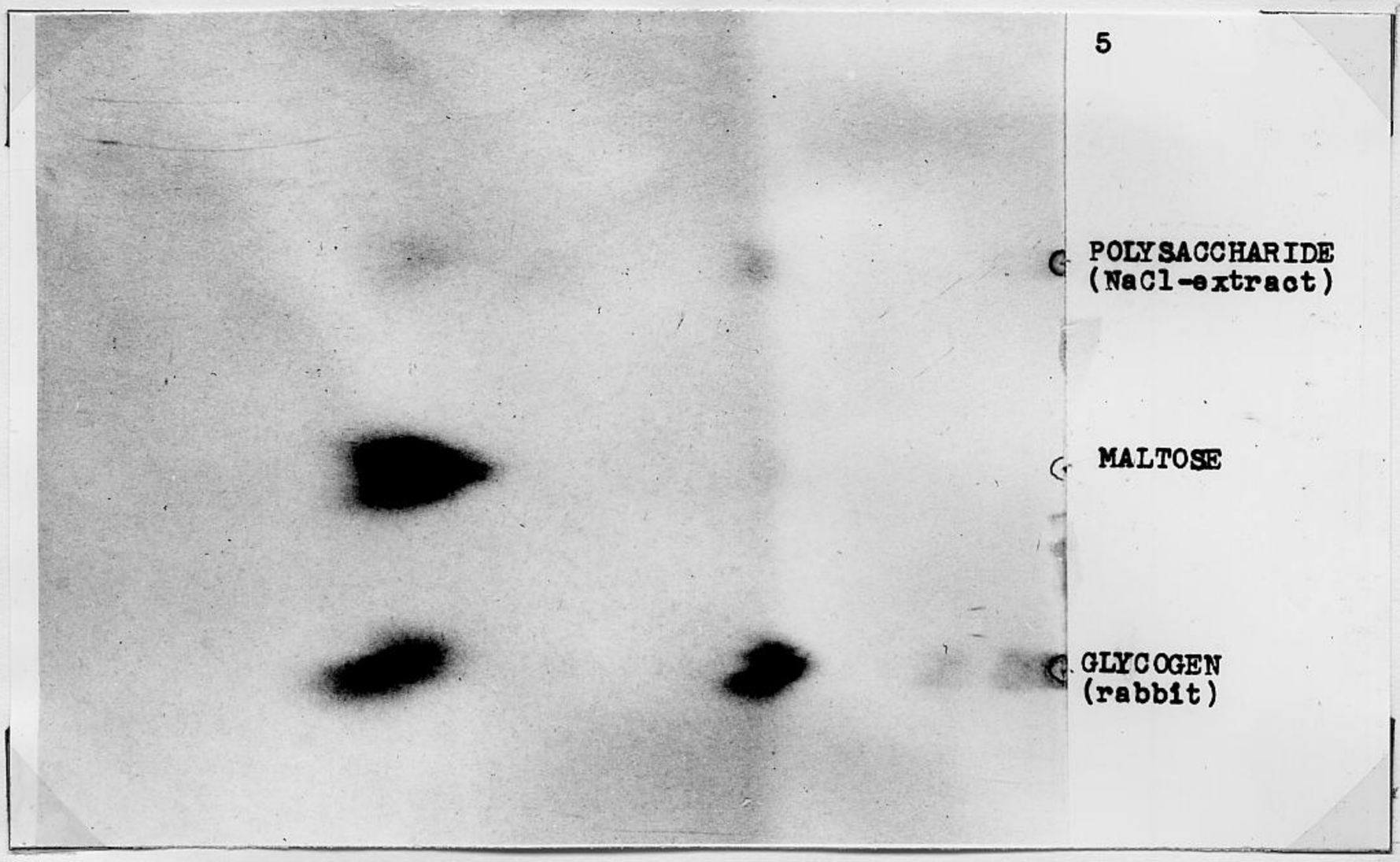


Fig. 7a

5 hours

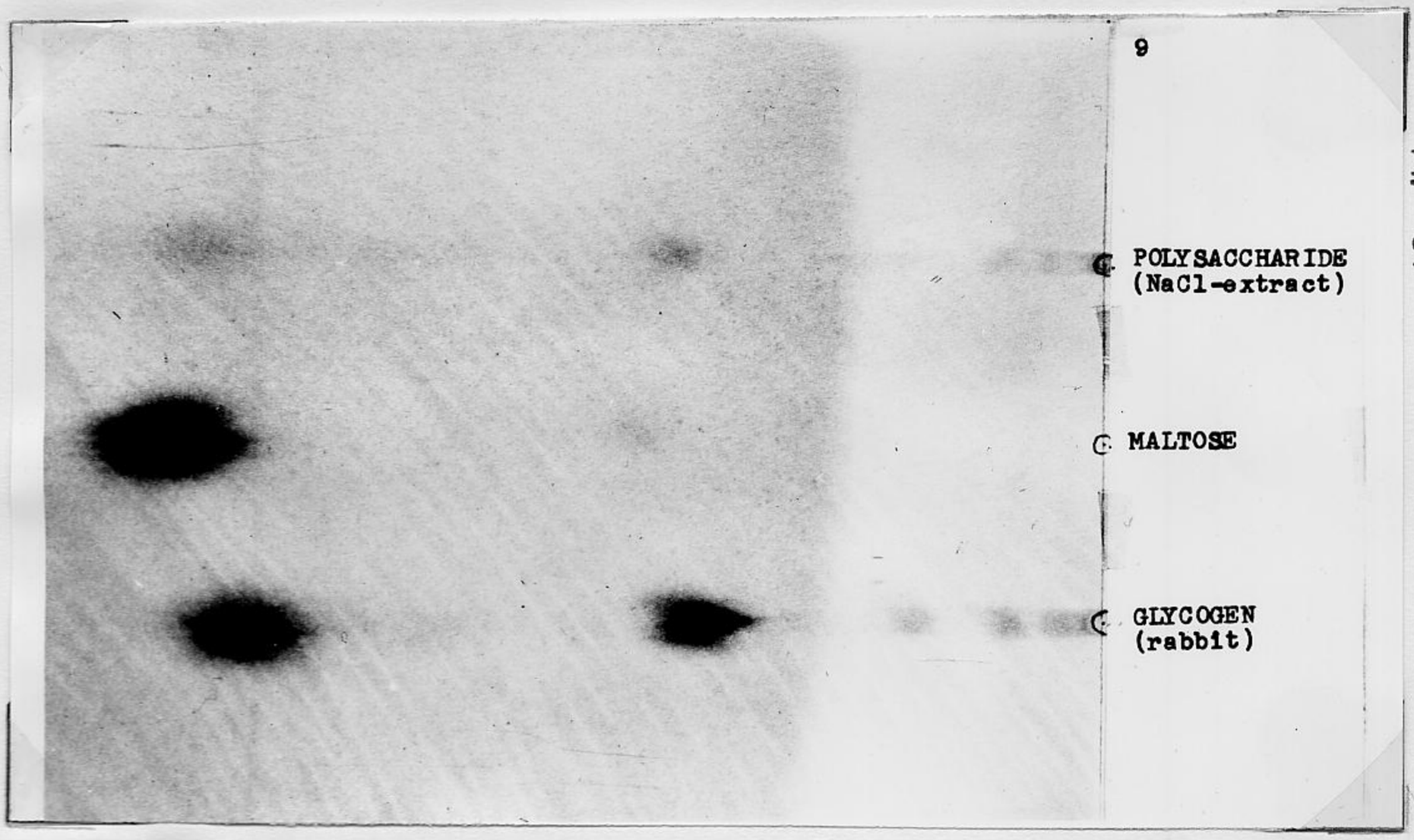


Fig. 7b

9 hours

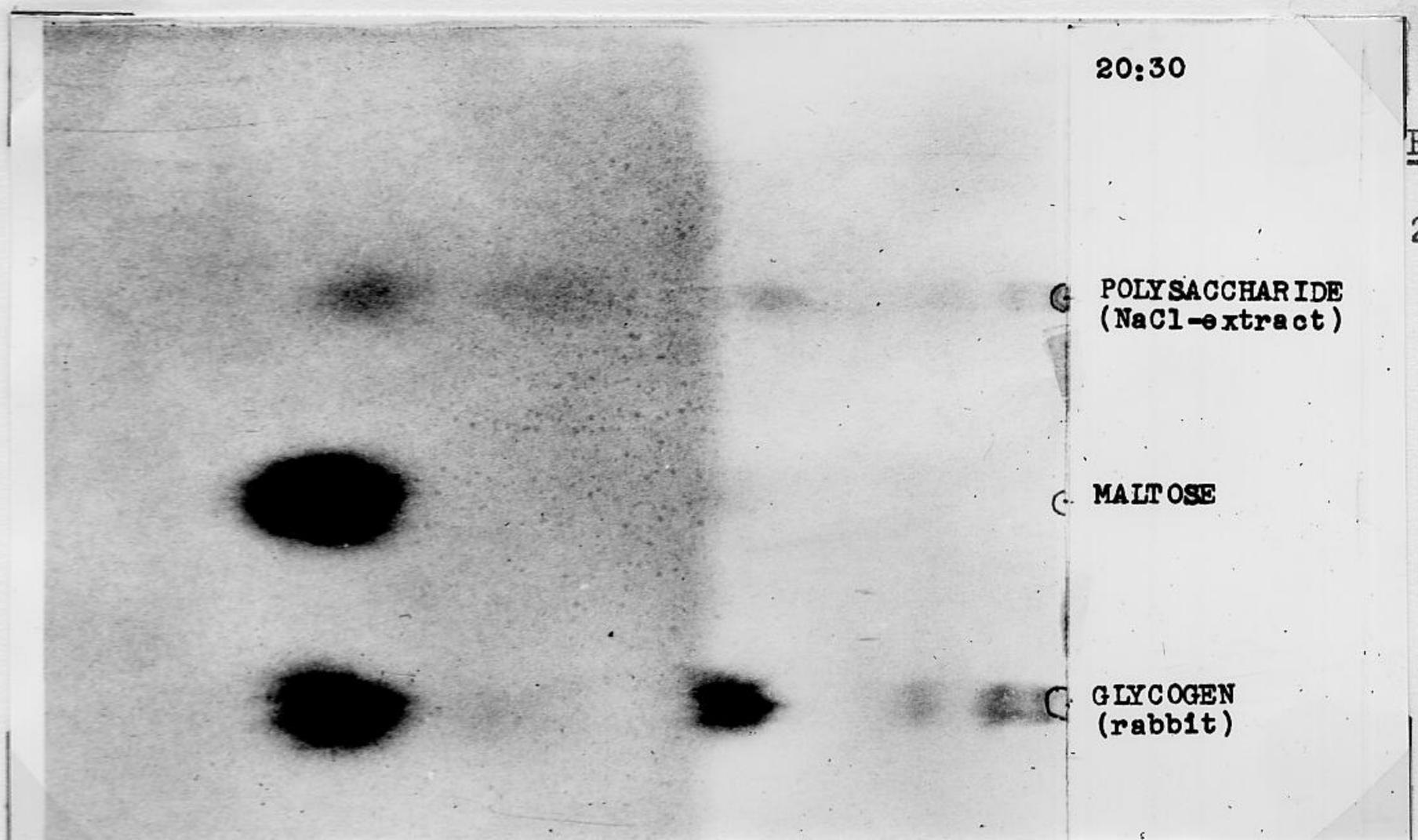


Fig. 7c

20.5 hours

TABLE II

PERCENTAGE OF MALTOSE LIBERATED DURING ENZYMATIC HYDROLYSIS

Time of hydrolysis (hrs)	Polysaccharide II		Reference rabbit glycogen	
	∝-amylase	β-amylase	∝-amylase	β-amylase
4	35	28	71.5	58
6	39.5	28	88.5	60.5
8	47	31.5	77	55
10	47	25	77	51
24	18.5	28	71	70

EXPERIMENTAL

Source of Material

Apparently healthy T. hydatigena cysts obtained from infected sheep were emptied of their fluid after excising the scolices. The white membranes were washed with tap water several times and stored at -25° until extracted.

Isolation of the Polysaccharide Component

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

Approximately 500 g of frozen cyst membranes were allowed to thaw, and then were homogenized in a Waring blendor and freeze-dried in a Virtis lyophilizer. The air dry tissues (41.266 g) were extracted with acetone (300 ml) by homogenization in the Waring blendor for 4 minutes. The resulting suspension was centrifuged in anMSE refrigerated centrifuge at 2300 r.p.m. for 20 minutes. Acetone extraction of the tissues was repeated two more times in the same manner. The tissue residue (residue A) was then extracted twice, in the same manner, with 100 ml portions of methanol-chloroform (3:1 v/v). The resulting lipid-free tissue was lyophilized and dried in a vacuum desiccator over NaOH pellets and P_2O_5.

30.4611 g of the dry lipid-free tissue (residue B) was extracted with toluene-saturated 2 M NaCl (600 ml) for 24 hours on a mechanical

shaker. Toluene was added to prevent bacterial growth. After centrifugation, the saline extract was stored at -25°, and the tissue residue was subjected to another similar saline extraction. The tissue residue finally left (residue C) was dialyzed against running tap water for 72 hours, freeze-dried and stored in the cold.

Both saline extracts were combined, dialyzed against running tap water for 72 hours, and the salt-free solution was concentrated to 300 ml by lyophilization. The concentrate was rendered 2 M with NaCl, deproteinized at room temperature by repeated shakings (26 x) with about 60 ml portions of Sevag's reagent (chloroform-amyl alcohol, 9:1 v/v) until no more gel-like material appeared at the interphase. The protein-free saline solution was then dialyzed for 72 hours against running tap water and freeze-dried. The fluffy white material obtained (0.6724 g) was designated as Extract I.

Residue C (11.5 g) was extracted with N NaOH (300 ml) at room temperature for 24 hours with continuous mechanical stirring. The sodium hydroxide extract was neutralized to litmus with glacial acetic acid and dialyzed for 70 hours against running tap water. The dialyzed solution was then concentrated to 200 ml by lyophilization, rendered 2 M with NaCl and deproteinized by repeated shakings (24 x) with Sevag's reagent. The protein-free solution was dialyzed against running tap water for 50 hours and freeze-dried to yield 1.0665 g of an extremely fluffy white residue designated

Extract II. Extracts I and II were stored in tightly stoppered containers at -25°.

Ultraviolet Analysis

Samples (1.1 ± 0.1 mg) from Extracts I and II, which were previously dried *in vacuo* over P_2O_5 at 60°C., were dissolved in deionized distilled water (5.00 ml). The solutions were transferred into quartz cells of 1.00 cm. pathlength, and the ultraviolet spectra (200 - 420 mμ) were determined in a Unicam SP 500 spectrophotometer, using the solvent as a blank.

Infrared Analysis

A dried sample (2.8 ± 0.1 mg) of Extract I was dissolved with 350 mg of potassium bromide (AnalaR) in 25 ml of deionized distilled water. The solution was well stirred, frozen and lyophilized. The mixture ready for pressing into a disc, was sent to the Chemistry Department of the University of Birmingham for analysis. The infrared spectrum was recorded on a Perkin-Elmer infrared spectrophotometer Model 21.

Hydrolyses

12 mg of each polysaccharide material (Extracts I and II) was hydrolyzed with 1 ml of 2 N H_2SO_4 at 100°C, for 4 hours. Hydrolyses were effected in small pyrex tubes fitted with air condensers. The hydrolyzates were then neutralized with $BaCO_3$ to pH 7, centrifuged and the clear supernatants lyophilized.

Paper Chromatographic Analysis

Approximately 1% solutions of all hydrolyzates were prepared. Glucuronic acid (potassium salt), glucosamine (hydrochloride salt), galactose, mannose and glucose were used as reference standards.

All paper chromatographic analyses were performed on Whatman No. 1 paper, using the descending technique.

After application of the spots, the chromatograms were developed using the organic phase of one of the following solvent systems:

1. 1-butanol-acetic acid-water (4:1:5 v/v) - 72 hours.
2. 1-butanol-pyridine-water (9:5:8 v/v) - 48 hours.
3. ethyl acetate-pyridine-water (2:1:2 v/v) . 12 hours.

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

1. aniline hydrogen phthalate for reducing sugars.[7]
2. 0.05% ninhydrin (AnalaR) in ethanol[8] for certain aminosugars.
3. silver nitrate[9]; 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% $Na_2S_2O_3$ solution followed by thorough rinsing with water.

Paper Ionophoretic Analysis

Paper Ionophoretic analysis was effected on strips (3 x 30 cm) of Whatman No. 3 MM paper in a Spinco-Durrum electrophoresis apparatus. 0.2 M - boric acid - borate buffer, pH 9.2, was used.

Solutions (0.1% - 0.2% in M NaCl) of the polysaccharide Extracts I and II and rabbit liver glycogen (as reference standard) were prepared. A total of 100 - 150 µl of each solution was applied. Ionophoresis was conducted at 150 v. for 4½ hours, and the polysaccharide spots were detected on the paper ionophoretograms with the periodic acid staining technique.[10]

Optical Rotation

The polysaccharide Extracts I and II 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.

Quantitative Determination of Glucose-Orcinol-Sulfuric-Acid Reaction[11]

Dry samples (1.2 - 1.3 mg) of Extracts I and II were hydrolyzed with 3 N H_2SO_4 at 100°C for 2, 2½, 3, 3½, and 4 hours. All hydrolyses were effected in glass-stoppered tubes in a boiling water bath. Each hydrolyzate was cooled, transferred quantitatively to a 10 ml volumetric flask and diluted to the mark using rinsings of the hydrolysis tube. The hydrolyzates were colourless and quite clear, and therefore, filtration was not necessary.

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 of 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, the tubes were kept immersed in the ice bath. After 15 minutes, the solutions were thoroughly mixed 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 mμ in 1 cm cells.

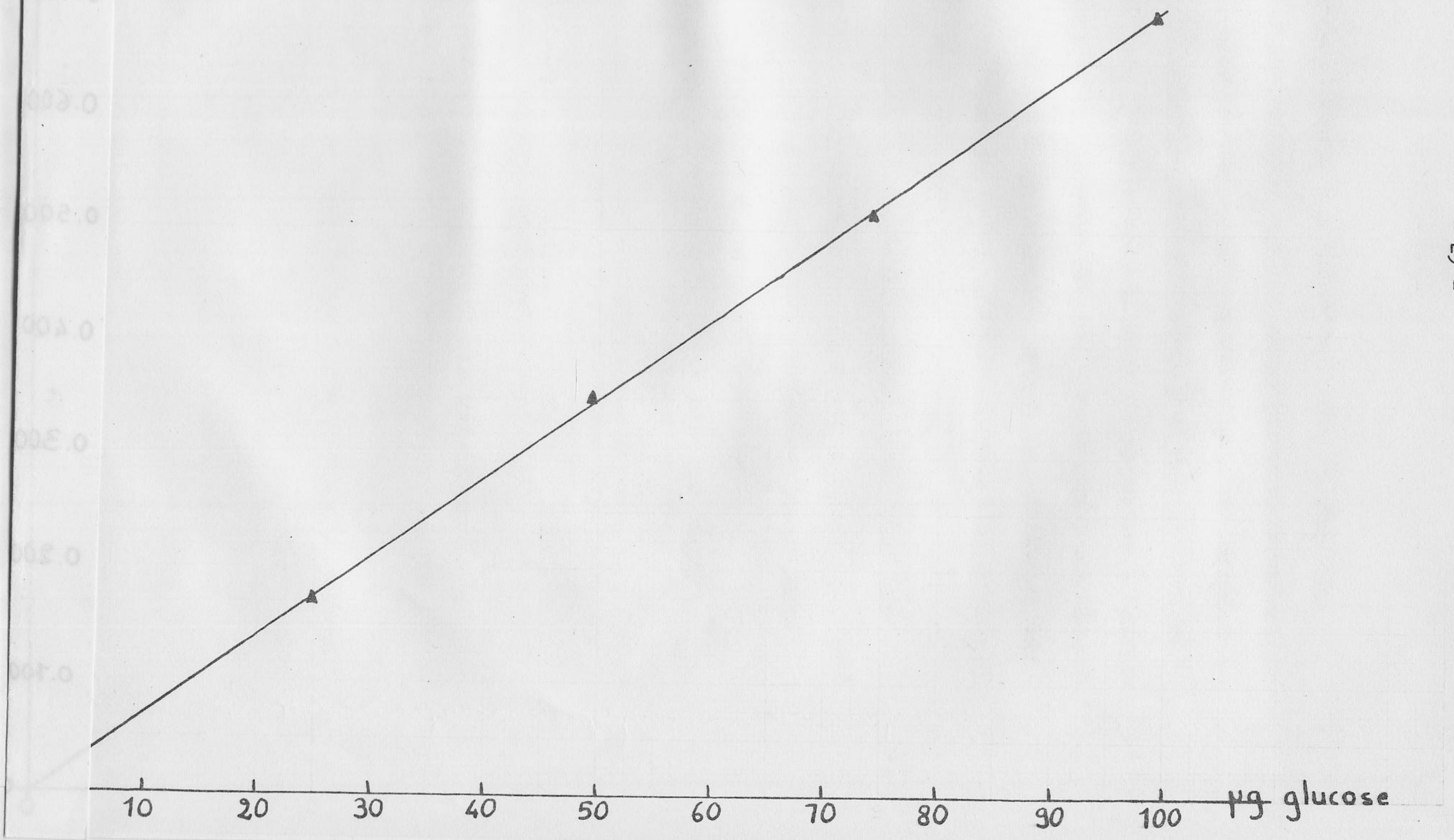
Standards of 0, 25, 50, 75 and 100 μg glucose were treated under the same conditions. The glucose content of the hydrolyzates was estimated from a calibration curve (Fig. 8) after subtraction of the sample blank reading.

Determination of the Absorption Maxima of the Iodine Complex[12]

Dry samples (1.8 mg) of the polysaccharide fractions I and II and of rabbit liver glycogen (reference standard) were dissolved each in a solution (4.0 ml) containing 0.20% iodine and 1.97% potassium iodide. The absorption maxima of the complexes produced were determined both in the ultraviolet region (240 - 420 mμ) and in the visible range (350 - 750 mμ).

FIG. 8 : ORCINOL - H_2SO_4 REACTION

Calibration Curve for Hexoses



Elementary Analysis

Elementary analysis for nitrogen, sulfur and halogens was conducted on samples of polysaccharide fractions I and II employing the sodium fusion method. Qualitative analysis was performed on an aqueous solution of the fusion product, using the conventional Prussian Blue, lead sulfide and silver nitrate tests. While sulfur and halogens could not be detected in polysaccharides I and II, both fractions gave a positive test for nitrogen.

Determination of Uronic Acid[13]

0.01% solutions of hyaluronic acid, rabbit glycogen and polysaccharide I were prepared.

To 1 ml of each solution, 6 ml of 98% sulfuric acid (B.D.H., Microanalytical Reagent) was added with cooling in an ice bath. The reaction mixture was then heated for 20 minutes in a boiling water bath and then cooled. 0.2 ml of a 0.1% ethanolic solution of carbazole (recrystallized twice from benzene) was added, and the solutions were left to stand for 2 hours at room temperature. Optical density values of the colours developed were read at 350 - 600 mμ on Unicam SP 500 spectrophotometer.

Enzymatic Hydrolysis

A. Qualitative Analysis

1. Amylolysis with β-amylase

Reference rabbit liver glycogen and polysaccharide I were incubated at a concentration of 5 mg/ml in 0.2 M acetate buffer

(pH 4.71) at 30 ± 0.5° with β-amylase (5 mg/ml). The enzymatic hydrolyzates were analyzed by paper chromatography after 2,4,6,8,10 and 24 hours using maltose as a reference standard. The paper chromatograms were developped with the organic phase of 1-butanol-acetic acid-water (4:1:5 v/v).

2. Amylolysis with α-amylase

Reference rabbit liver glycogen and polysaccharide I were incubated each at a concentration of 5 mg/ml in buffered saline solution (0.03 M phosphate, 0.04 M NaCl); pH 6.6, at 30 ± 0.5°, with α-amylase (5 mg/ml). The enzymatic hydrolyzates were analyzed by paper chromatography as previously mentioned after 3,5,7,9,11 and 20.5 hours.

B. Quantitative Analysis

Reference rabbit liver glycogen and polysaccharide II were dried in vacuo at 60° for 4 hours. 10.0 mg samples of reference glycogen and polysaccharide II were incubated separately each with 10.17 mg. of α-amylase in 2 ml of phosphate-buffered saline as previously described at 30°. At the same time incubation of reference rabbit glycogen and polysaccharide II was conducted with β-amylase under the same conditions using the same concentrations as above and the appropriate buffer. Aliquots of the enzymatic hydrolyzates were analyzed quantitatively for reducing sugars after 4,6,8,10 and 24 hours. In the analysis the control solutions used consisted of reference rabbit glycogen and polysaccharide II in phosphate-buffered saline and acetate buffer, solution of α-amylase in buffered saline and solution of

β -amylase in acetate buffer. All control solutions were incubated under the same conditions as the substrates.

The reducing sugars liberated were determined quantitatively using the Nelson's colorimetric modification[35] of Somogyi's method.

In a typical run, 0.02 ml aliquot of each enzymatic hydrolyzate was diluted to 1 ml with deionized distilled water and 1 ml of the low-alkalinity copper reagent was added. The tubes were heated for 10 minutes in a vigorously boiling water bath and then cooled. 1 ml of arsenomolybdate reagent was added, and when all the cuprous oxide dissolved, 10 ml of deionized distilled water was introduced and the solutions were left to stand for 15 minutes. The absorbances were read at 500 mu in a Unicam SP 600 spectrophotometer. The blanks, controls and reference standard tubes containing 20, 40, 60, 80 and 100 ug of maltose were treated in the same way. The reducing sugar content of the enzymatic hydrolyzates was calculated using the calibration curve (Fig. 9).

FIG. 8 : ORCINOL - H_2SO_4 REACTION

Calibration Curve for Hexoses

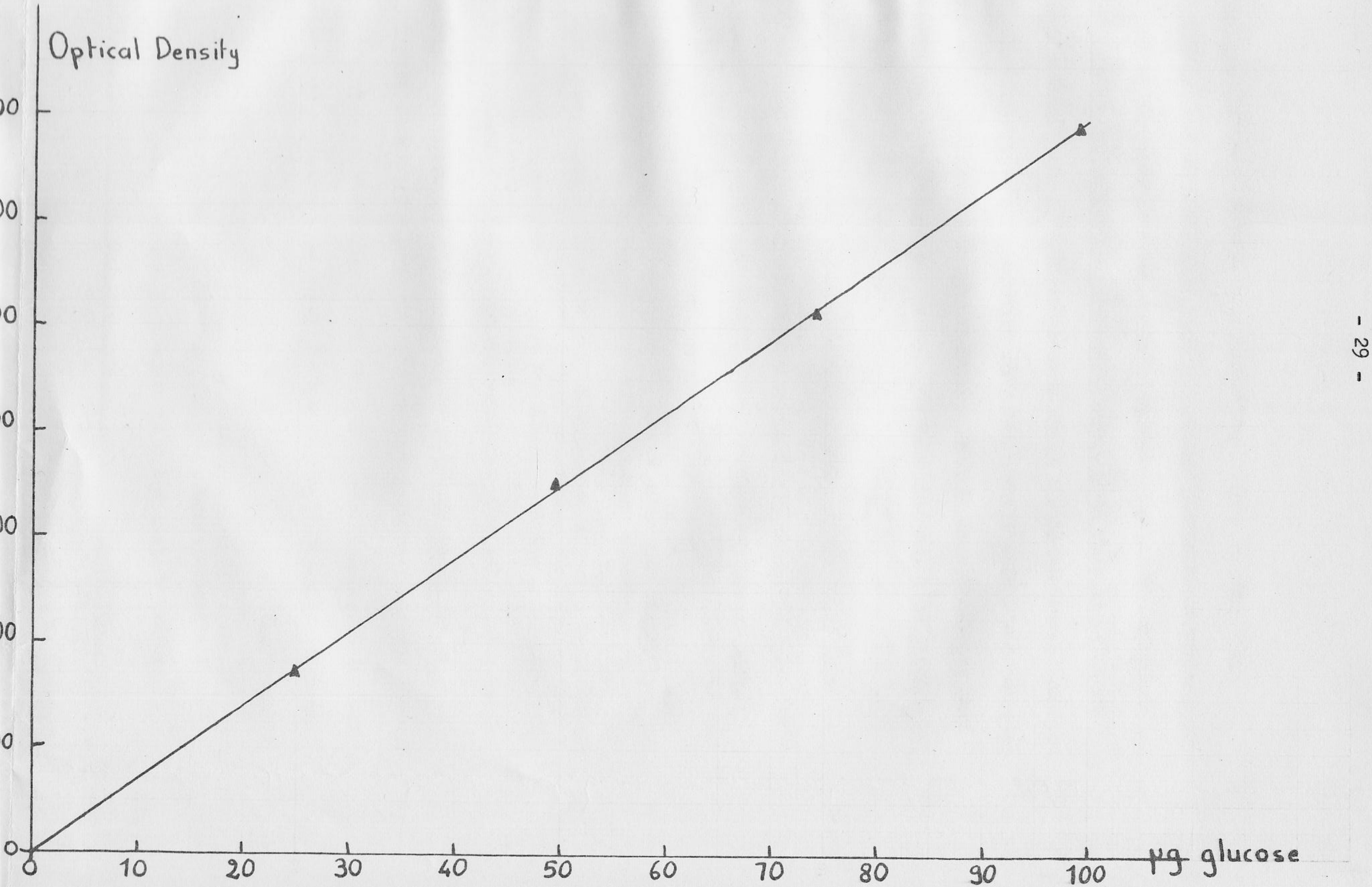
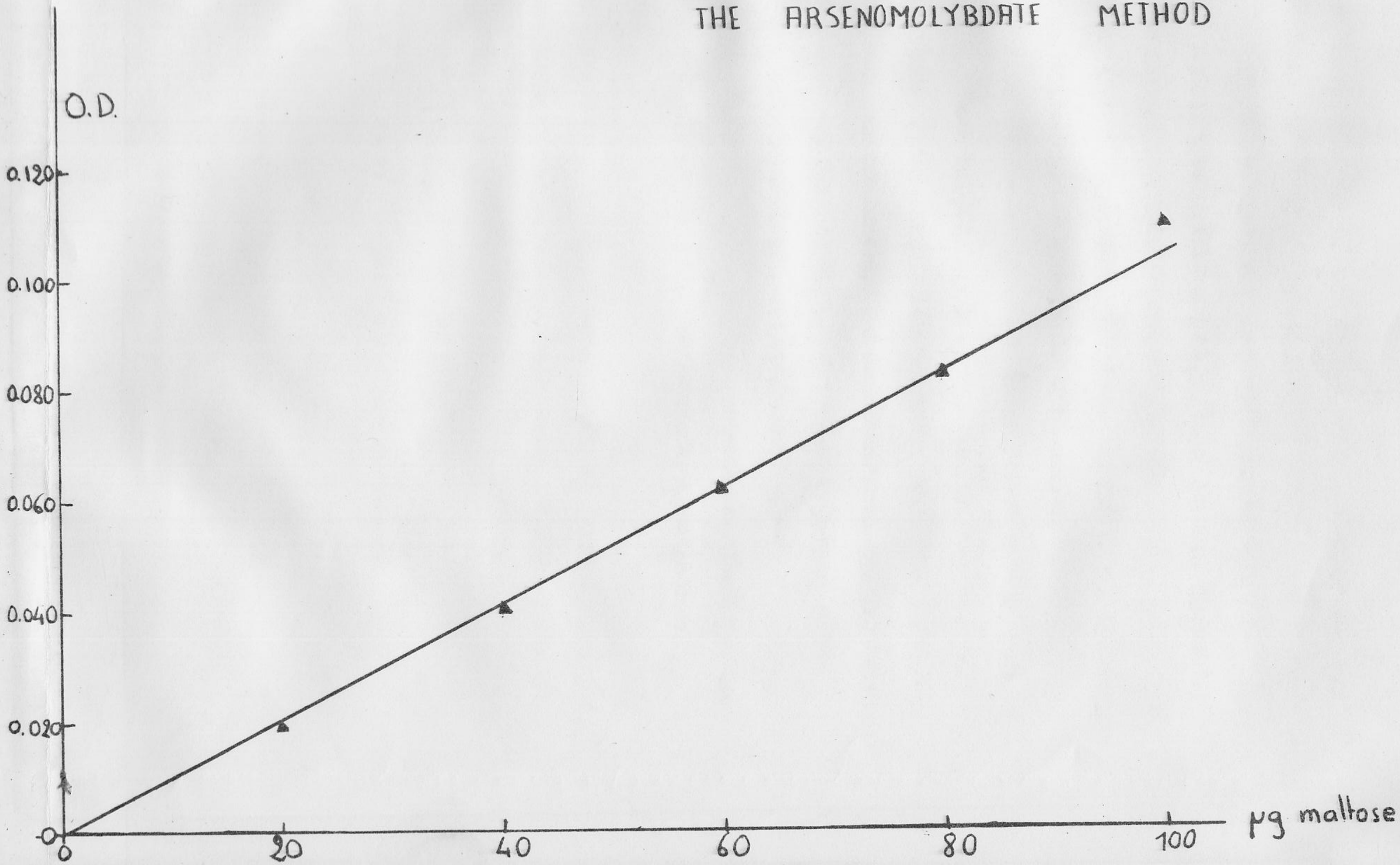


FIG. 9 : MALTOSE DETERMINATION BY THE ARSENOMOLYBDATE METHOD



LIST OF REFERENCES

1. Chandler, A.C., Introduction to Parasitology, 9th Ed., John Wiley and Sons Inc., New York, 1955, p. 329.

2. Sweatman, G.K. and Plummer, P.J.G., Canad. J. Zool., 35, 93 (1957).

3. Kilejian, A., Sauer, K., and Schwabe, C.W., Exper. Parasitol., 12, 377 (1962).

4. Orr, S.F.D., Biochim. Biophys. Acta, 14, 173 (1954).

5. Mathews, M.B., Nature, 181, 421 (1959).

6. Wolfrom, M.L. and Juliano, B.O., J. Am. Chem. Soc., 82, 1673 and 2588 (1960).

7. Partridge, S.M., Nature, 164, 443 (1949).

8. Payne, W.J. and Kieber, R., Arch. Biochem. Biophys; 52, 1 (1954).

9. Trevelyan, W.E., Procter, D.P., and Harrison, J.S., Nature, 166, 444 (1950).

10. Block, R.J., Durrum, E.L., and Zweig, G., Paper Chromatography and Paper Electrophoresis, 2nd Ed., Acad. Press Inc., New York, 1958, p. 578.

11. Svennerholm, L., J. Neurochem., 1, 42 (1956).

12. Barker, S.A., Stacey, M., and Tipper, D.J. Clin. Chim. Acta, 4, 861 (1959).

13. Dische, Z., J. Biol. Chem., 167, 189 (1947).

14. Sevag, M.G., Biochem. Z., 273, 419 (1934).

15. Davies, D.A.L., Biochem. J., 59, 696 (1955).

16. Davies, D.A.L., Morgan, W.T.J., and Mosimann, W., Biochem. J., 56, 572 (1954).

17. Davies, D.A.L., Morgan, W.T.J., and Record, B.R., Biochem. J., 60, 290 (1955).

18. Heidelberger, M., Dische, Z., Neely, W.B., and Wolfrom, M.L., J. Am. Chem. Soc., 77, 3511 (1955).

19. Fuller, K.W. and Northcote, D.H., Biochem. J., 64,657 (1956).

20. Stimson, M.M. and O'Donnell, M.J., J. Am. Chem. Soc., 74, 1805 (1952).

21. Pearson, F.G., Marchessault, R.H., and Liang, C.Y., J. Polymer Sci., 43, 101 (1960).

22. Bellamy, L.J., The Infra-red Spectra of Complex Molecules, 2nd Ed., Methuen and Co. Ltd., London, 1962.

23. Whiffen, D.H., Chem. and Ind., 129 (1957).

24. Barker, S.A., Bourne, E.J., and Whiffen, D.H., Methods of Biochemical Analysis, Vol. 3, Acad. Press, New York, (1956), p. 213.

25. Dische, Z., Methods in Carbohydrate Chemistry, Vol. I, Academic Press, New York, (1962), p. 497.

26. Stutz, E. and Deuel, H., Helv. Chim. Acta, 39, 2126 (1956).

27. Jermyn, M.A. and Isherwood, F.A., Biochem. J., 44, 402 (1949).

28. Chargaff, F., Levine, C., and Green, G., J. Biol. Chem., 175, 67 (1948).

29. Partridge, S.M., Nature, 158, 270 (1946).

30. Partridge, S.M., Biochem. J., 42, 238 (1948).

31. Vasseur, E., Acta Chem. Scand., 2, 693 (1948).

32. Rundle, R.E. and French, D., J. Am. Chem. Soc., 65, 1707 (1943).

33. Baldwin, R.R., Bear, R.S., and Rundle, R.E., J. Am. Chem. Soc., 66, 111 (1944).

34. Whelan, W.J. and Bailey, J.M., Biochem. J., 58, 560 (1954).

35. Hodge, J.E. and Hofreiter, B.T., Methods in Carbohydrate Chemistry, Vol. I, Academic Press, New York, (1962), p. 380.

36. Manners, D.J., Advances in Carbohydrate Chemistry, Vol. 12, Academic Press, New York, (1952), p. 261.