INVESTIGATIONS IN THE PREPARATION OF URONIC ACID SULPHATES

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by

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ABSTRACT

Many naturally occurring carbohydrates contain sulphated uronic acid units. Very little is known about the structural properties of these sulphates uronic acid units. This work was concerned with the investigation of some methods of preparing the monosaccharide sulphates with a view to finding a method suitable for preparing model compounds.

Sulphation reactions were carried out with D-glucuronic acid and D-galacturonic acid using three sulphating agents. Chlorosulphonic acid and pyridine sulphur trioxide complex gave some yield of uronic acid sulphate but no satisfactory method of purification was found. The unreacted starting material accompanied the sulphate at all stages of work up. This effect may also have been complicated by hydrolysis of the uronic acid sulphate under the conditions used. Sulphuryl chloride did not appear to sulphate the uronic acids with the conditions used, except possibly, in very small amounts.

Pectin and alginic acid when treated with sulphuryl chloride however did give a product with an appreciable sulphur and chlorine content and which probably contained a chloro-sulphate of the polyuronic acid.

A pure sample of D-mannuronic acid was prepared and the NMR spectra of D-glucuronic acid, D-galacturonic acid and D-mannuronic acid obtained.

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This is to certify that all the experimental work described in this thesis was carried out under my supervision during the sessions 1964-1969.

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C O N T E N T S

INTRODUCTION

Polysaccharides and their derivatives are widely distributed in both the plant and animal kingdoms and have been shown to play a fundamental role in the biochemistry of living tissues. These important biological functions have required much detailed research into the structure of these compounds to determine the active principles involved. Most of the earlier work was carried out on starch and cellulose which were readily available: more recent techniques of investigation have enabled researches into other types of polysaccharides such as plant gums and mucilages, pectins, hemicullulose and immune-polysaccharides, to proceed revealing the high degree of complexity involved in the structure of these compounds.

Sulphated polysaccharides occur in both the animal and plant kingdoms. In the plant kingdom many of the marine algae contain polysaccharide sulphates, the most abundant of these are the brown seaweeds (Phacophycea), many of which contain the sulphated polysaccharide fucoidin. The red seaweeds (Rhodophycea), Chondus crispus and Gigartina stellata give the carrageenin polysaccharides and Iridea laminarioides contains galactan sulphates. Agar-Agar, a sulphated polysaccharide of commercial importance is extracted from R Floridea. Some of the green seaweeds (Chlorophycea) also contain sulphated polysaccharides. Animal tissues also contain polysaccharide sulphates such as fucoidin, found in the jelly coat of sea urchin eggs, and galactan sulphates which are found in brain lipids. Carbohydrate sulphates in loose combination with proteins, as salts, form viscous slimes which have lubricating and protective properties.

Uronic acids, their salts and derivatives, are also found in living tissue in association with polysaccharide sulphates. Alginic acid (poly mannuronic acid) is a major constituent of the brown algae pectic layers. Heparin, hyaluronic acid and the chondroitin sulphates, all having uronic acid units (glucuronic acid and iduronic acid) as part of their structural segments, are found in animal tissues nad have been found to have very powerful physiological effects.

Polysaccharide sulphates containing uronic acid units.

Glucuronic acid is found in a 1:1 ratio with substituted 2-amino-2-deoxy-D-glucose in the important and widespread glycans, heparin, chondroitin 4 and 6 sulphates and hyaluronic acid. Heparin is produced by mast cells and is widely distributed in the tissues of animals. It has a variety of biological rules such as the inhibition of ribonuclease, a clearing factor for blood lipid and as a blood anticoagulent. The latter is its most significant activity. Here it prevents blood coagulation by interfering with the conversion of prothrombin to thrombin. It has a commercial importance in pre-surgical medication to lessen the possibility of operative and post-operative thrombosis. Heparin is a heteroglycan sulphate believed to be an alternating copolymer of D-glucuronic acid units with 2-amino-2-deoxy-D-glucose units in a linear chain. For each four sugar units there are five or six sulphate groups. Two of these are combined with amine groups as sulphamic acids and the remainder with hydroxyl groups as sulphonic half esters. The anticoagulant activity in the intact polysaccharide is a function amongst other things of its sulphate content. The position of the sulphate half ester is not as yet established and additional investigation is required.

Chondroitin sulphates A and C are also polysaccharides containing an alternating linear copolymer of glucuronic acid and 2-acetamide-2-deoxy-D-galactopyranosyl monosulphate units linked (1→3) and (1→4) respectively. Neither chondroitin sulphate A nor C shows anticoagulant activity.

Hyaluronic acid, found in many animal tissues, contains equimolar amounts of D-glucuronic acid and 2-acetamide-2-deoxy-D-glucose. Hydrolysates of chondroitin sulphates A and C both contain D-glucuronic acid, 2-amino-2-deoxy-D-galactose, acetyl and sulphate residues in equimolar amounts. Heparin also yields equimolar amounts of D-glucuronic acid and 2-amino-2-deoxy-D-glucose.

Chondroitin sulphate D, isolated from shark cartilage by Suzuki[1], has an infra red spectrum identical with chondroitin sulphate C. The non identity of the two polysaccharides are shown by their difference as acceptors in enzymic desulphation and by the higher sulphur content of chondroitin sulphate D (7.6%). Suzuki, during his examination of chondroitin D, isolated an unsaturated disulphated disaccharide from which a mono sulphated disaccharide was obtained and oxidation studies indicated the presence of a C2 or C3 sulphated uronic acid.

Heparin fragments have also been shown to contain C2 and C3 sulphated glucuronic acid as well as amino and C2 sulphated residues on the hexose fragment.

Polysaccharide sulphate structure, methods used and problems involved.

The increasing interest in glyco proteins and mucopolysaccharides of biological importance has increased the need for

structural information and hence the need for improved methods of structural analysis. At present no one method will give all the information required and what information is available is usually obtained by inference from several methods of attack.

One of the major difficulties in polysaccharide analysis is obtaining pure samples from complex mixtures of natural products which often require a chemical treatment to make them suitable for separation as for example the use of hydrolysis techniques to obtain soluble material for separation.

Separation of the mixture components is then effected by methods such as precipitation, solvent extraction and partition or column chromatography.

Most techniques give a pure product only with repeated application or when used in combination with other techniques resulting in small yields of the desired product.

The comparatively recent development of the method of 'gel filtration' into 'gel permeation chromatography' by Moore[2] and the introduction of commercially available equipment has greatly facilitated the range of separations available. A wide range of 'gel' packing materials is now available, such as Sephadex, Bio-gel (cross-linked polysaccharides or polyacrylamide respectively) and their ionic derivatives (diethylamino-, carboxymethyl-) and porous glass (Bio-Glass, Porasil). With these elution techniques a number of factors affect the elution volume of the species of interest but by a choice of suitable 'gel' and eluant system it is often possible to obtain a complete separation.

The method uses as a separation parameter the hydrodynamic volume of the species present and with the development of suitable

calibration techniques[3] can be used to obtain molecular weight average data and to study the molecular weight distribution characteristics of polysaccharides.

Undegraded hyaluronic acid-protein complex has been obtained together with a description of the molecular weight distribution of hyaluronic acid by G.P.C. ('gel permeation chromatography').on agarose (polysaccharide)column[4,5].

A generally applicable definition of homogeneity in polysaccharide preparations is difficult because in addition to the polydisperse nature of the molecular weight distribution the concept of a structurally repeating unit cannot be used. Usually the evidence can only lead to the conclusion that certain elements of structure are present and only rarely can all the units and their sequences be defined. With highly branched structures random or semi-random distribution of the chains prevents the identification of more than an average scheme o f the structural features.

In recent years quantitative analysis of the component sugars of polysaccharides has become more reliable. Combined separation and hydrolysis of products gives the most meaningful results but the problem of getting the quantitative liberation of individual units from the polymer has not been solved, particularly for molecules containing uronic acid residues.

The determination of the structure of sugar units in a polysaccharide chain has two main factors:

(a) linkage type; (b) linkage sequence.

Linkage sequence and the mono saccharide unit sequence is a complex problem with at present no general methods of solution. A promising area being developed is the method of sequence and

unit linkage degradation by exo and endo enzymes[6]. Usually specific information can only be obtained in conjunction with chemical reactions, and is primarily effective with highly branched molecules.

The well established methods of methylation, hydrolysis and oxidation can be used to determine the unit linkage and subsequently the position of substituents but no general procedure is available. Hoffman degradation has also been used by Kolchetokov[7] to cleave the glucuronosidic link in polysaccharides to obtain selective splitting of the chain. The cleavage of the glucuronosidic link in five polysaccharides containing uronic acid units by the formation of the amide and Hoffman degradation was studied and under the conditions used the 2, 3 and 4 glucopyranose structure was retained.

With sulphated polysaccharides determination of the glycosidic linkages is more difficult, the sulphate group increases the solubility of the methylated polysaccharide making purification more difficult. Methylation is also made more difficult by the presence of the group and on hydrolysis the sulphate group may be removed preventing the definition of the glycoside link. The use of an esterified sulphate group improves solubility problems but the sulphate group is still susceptible to hydrolysis[8]. The position of the sulphate group can not be fixed by any general method but has to be inferred from information obtained from other parts of the molecule together with infra red spectral information[9,10,11,12], desulphation[13-20], degradation products[21,22] and possibly alkaline hydrolysis rates.

The problem is further complicated in that the structural units have to be identified as well as the substitution position within the unit. Periodate oxidations of polysaccharides[23] can give information on the position of glycosidic linkages and also the degree of branching at the end groups. Complications occur from the presence of a sulphate group[24,25] in that the theoretical periodate uptake may not be attained and ionic sulphate released.

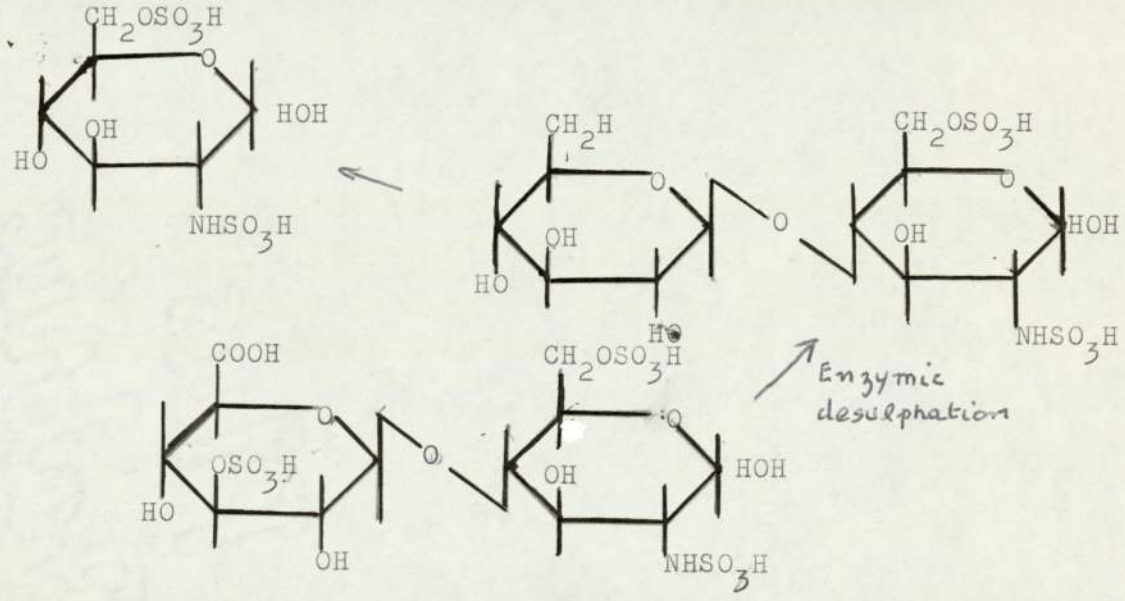
Rates of periodate oxidation of acid mucopolysaccharides have also been related to structural units by Scott and Harbinson[26] who used periodate in the presence of 0.2 molar sodium perchlorate to oxidise the chondroitin sulphates A, B and C, some mucous and lung heparins, hyaluronidates, seaweed and plant glycurans. They found that uronic acid moieties were oxidised slowly, the L uronic acids more rapidly than the D uronic acids whilst hexosamine, carboxyl and sulphate were not attacked. Percival and Soutar[27] found no difference in acid hydrolysis rates for the sulphates of glucose and galactose. Clancy and Turvey[22] studied the acidic hydrolysis rates of methyl-glycosides and methyl-glycoside 3 and 6 sulphates and found that the C6 sulphates were more stable than the C3 sulphates. It was suggested that the stabilising effect is due to the C6 sulphates being equatorial which increases the stability of the chain conformation and slows down the hydrolysis which takes place through the half chair conformation. Rees[29] confirmed that the acidic hydrolysis rates could be useful for the characterisation of single sulphates and could be a guide to the types of ester present in compounds with several sulphate groups.

Carbohydrate degradation in alkaline solution under controlled conditions can be used in certain cases to progressively degrade the polysaccharide in a known way, e.g. 3-O-substituted and 4-O-substituted sugars give, in calcium or barium hydroxide under controlled conditions, 3-deoxy aldonic acids. Degradation proceeds from the reducing and via enolisation and beta elimination reaction forming a new reducing unit. Whistler and Corbett[30] used an extension to branch point determination using the polysaccharide guran. This method really requires rapid analytical methods.

Enzymic degradation can be very useful in the analysis of muco polysaccharides in that reaction conditions are usually mild and the enzymic reactions can be highly specific enabling the reaction products to be directly related to the polysaccharide structural units. The method together with information from chemical reactions can potentially give specific information for both sequence and unit analysis. The major limiting factor at the present time is the need to find and adapt suitable enzymes. It should be pointed out however that different methods can lead to conflicting results as for instance in the assignment of sulphate substituents to units in the heparin molecule. Wolfram et al[21], using a periodate oxidation technique, concluded that all the sulphate was present in the glucosamine units. They oxidised the sodium salt of heparin with periodate to destroy any O-glucuronic acid residues, reduced the product with borohydride and removed amine by treatment with nitrous acid finally obtaining 2-5 anhydro-aldehydo-D-mannose 6 sulphate which was isolated as the

brucinium salt and identified by comparison. Their results indicate that of the generally accepted five sulphate groups present per tetra saccharide unit two are on the C6 of the 2-amino-2-deoxy-D-glucose units and that the D-glucuronic acid residues are not sulphated. The essence of this result is that to attack the glucuronic acid unit it is necessary to have a glycol group present and it is suggested that the only available position for sulphation in the uronic acid unit is at C3. The absence of glucuronic acid residues in t he periodate oxidation product therefore precludes the uronic acid being sulphated. Dietrich[31], however, using an enzymic degradation of heparin found sulphate to be present in the uronic acid residue as did Danishefsky[22] using methylation and hydrolysis.

Dietrich used adapted cells of Flavoheparinium bacterium to degrade heparin. After incubation at 25°C in neutral suspension the product was prepared and freeze dried then purified by gel filtration on Sephadex G25. Dietrich found that five components were present in the product, three of which contained sulphate. Partial desulphation and further degradation to 2:6 glucosamine sulphate together with infra red analyses indicated that the three compounds were



From these results it was concluded that uronic acid units with sulphate substituted at the C3 position were present in the heparin molecule. Danishefsky et al investigated the position of O sulphates in heparin by forming the acetamide derivative, permethylating with dimethyl sulphate, esterifying the carboxyl then reducing and hydrolysing the polysaccharide followed by examination of the fragments obtained by comparison of crystalline derivatives, chromatography and colour reactions. The fragments found were 3-O-methyl D glucose, 2,3 di-O-methyl-D-glucose, 3-O-methyl D glucosamine, glucosamine and 3,6 di-O-methyl-D-glucosamine and based on the amounts isolated they suggest that one third of the glucuronic acid moieties present in heparin are sulphated on C2.

If the sulphate is substituted on C2 of the glucuronic acid group then periodate oxidation of the uronic acid unit would be feasible in agreement with the findings of Wolfram et al. In addition there is the presence of iduronic acid in the heparin molecule[32] to be taken into account, as yet it is not known if this unit is sulphated or what its role is.

The use of gas-liquid chromatography for the separation and identification of sugars has also been usefully extended by the introduction of trimethylsilyl groups into sugar molecules to give volatile derivatives. Since the trimethylsilyl derivatives are also suitable for mass spectrometer analysis, they can also be used to provide additional confirmation of the skeletal structures of the sugar[33].

A problem still remians however in that suitable reference compounds are required to compare retention times.

Optical rotatory dispersion studies have also proved useful in determining the stereochemistry of asymmetric centres in hexoses using suitable derivatives (benzimidazole, quinoxaline, flavazole or anhydroosazone).[34]

Two problems which arise from the structural complexity of these sulphated materials are

(a) what are the structures of the carbohydrate moieties before the treatment necessary to analyse them, the usual treatment required to bring them into solution and to purify them from other residual substances can cause desulphation.

(b) what are the properties of the individual sulphated carbohydrate units?

Sulphated uronic acid units are found as link units in some physiologically important compounds such as heparin. Very little is known about either the clinical or chemical properties of these sub-units or what contribution they make to the properties of the whole compound.

A method of attempting to obtain this information is to prepare the sulphated carbohydrates as model compounds for investigation.

Preparation of sulphated carbohydrates.

Organic materials have been sulphated by reaction with sulphuric acid, oleum, sulphur trioxide, sulphuryl chloride and a sulphur trioxide complex with pyridine or other organic base. The last three reagents have been used, with some success, to sulphate carbohydrates.

Chlorosulphonic acid in pyridine was used by Chle[35] to sulphate 1,2-O-isopropylidene glucofuranose and 1,2:5,6-di-O-isopropylidene glucofuranose. The products, isolated as the barium or brucine salts, had sulphate residues on C6 in the first case and in the second at C3.

Using the same reagent Soda and Nagai[36] found that in the main substitution occurred at C6. Some products were also found with more than one sulphate residue.

Percival and Souter[37] used chlorosulphonic acid to sulphate galactose, 1,2:3,4-di-O-isopropylidene galactopyranose, methyl-glucoside and methyl-galactoside. They found that substitution occurred preferentially at the primary alcohol group when this group was available. Galactose sulphate was highly reducing and mutarotating so that the sulphate residue was attached to C2, C3, C4 or C6. The methyl-glucosides when hydrolysed with barium hydroxide formed a 3:6-anhydro sugar. These results indicate that the sulphate group was on C6 since if it was on C3 other compounds containing anhydro rings would have been expected to form.

A pyridine sulphur trioxide complex was used by Duff[38] to sulphate 3,4-O-isopropylidene-1,6-anhydro-D -galactose to obtain the 2-sulphate derivative. Glucose was also sulphated with this reagent to yield a monosulphate.

Peat et al[39] used pyridine sulphur trioxide for the direct sulphation of D-glucose, D-galactose, methyl α and β D-glucosides and methyl β D-galactoside. Glucose and galactose gave three monosulphates one of which was predominant. The major components were separated on a cellulose column and the recovered material

compared with glucose 6-(Barium sulphate), glucose 3-(barium sulphate) and galactose 6-(barium sulphate). In each case the major component was shown to be the C6 sulphate. An increase in time and temperature of reaction resulted in an increase of sulphated material and the formation of disulphates. Reaction of the free sugar with sulphur trioxide for 10 hours at 70°C gave traces of a trisulphate. Turvey and Clancy[40] did similar work but used ion exchange resin to separate the sulphate derivatives.

Turvey and Harris[41] have investigated the pyridine sulphur trioxide reaction for sulphating monosaccharides and their results indicate the presence of an equilibrium reaction so that complex rich areas in the reaction mixture yield disulphate and the formation of mono and di sulphates can be enhanced by suitable choice of reaction conditions.

Lloyd[42] used chlorosulphonic acid for the sulphation of 1,2,3,4 tetra-O-acetyl-β-D-glucose in the preparation of glucose-6-sulphate. The sulphate ester was separated from free sugar by column chromatography on cellulose. After removal of the acetyl groups by refluxing in acetic acid and further purification by cellulose column chromatography the sulphate ester obtained was compared with the product from direct sulphation of glucose. The two products had identical properties. Similar treatment of 1,3,4,tri-O-acetyl-N-acetyl-β-D glucosamine to obtain an N-acetyl glucoasmine 6-sulphate product which gave identical properties to the product obtained from the direct sulphation of N-acetyl-glucosamine.

Lloyd[43] also sulphated D-glucose, D-galactose, N-acetyl-glucosamine, N-acetyl-galactosamine and D mannose 6 sulphate by reaction with pyridine sulphur trioxide complex. Increased yields of sulphated saccharides were obtained by use of a drying agent, Drierite, in the reaction mixture. Purification was performed by zone electrophoresis in cellulose columns. Reference C6 sulphate compounds were also prepared from the appropriate acetyl or isopropylidene derivatives.

Results of infra-red spectrum analysis and an inability to form a triityl derivative showed that the sulphate group had reacted with the C6 hydroxyl group.

Dodgson and Spencer[44] used both chlorosulphonic acid and pyridine sulphur trioxide complex for the direct sulphation of glucose. They obtained 6-sulphate contaminated with two other sulphated products. Hydrolytic desulphation with hydrazine was slow for the C6 sulphate but rapid for the other two products. Guiseley and Ruoff[45] sulphated glucose using pyridine sulphur trioxide complex and NN-dimethylformamide as solvent when it was found that the amount of disulphate formed was less than when using pyridine as solvent. When methyl-4,6-O-benzylidine-D-glucopyranoside was sulphated in this way it was shown that the sulphate group entered position C2 by comparison with the C2 sulphate prepared from methyl 4,6-O-benzylidine glucose 3-nitrate.

Wolfram and Shen[46] used sulphur trioxide-NN-dimethylformamide complex in NN dimethylformamide solvent to sulphate chitosan. A trisulphate of methyl-2-amino-2-deoxy-D-glucopyranoside hydrochloride was also prepared by the same workers using chloro-

sulphonic acid in pyridine at 60°C.

Grant[47] used pyridine sulphur trioxide complex to sulphate 1,2:3,6-di-O-isopropylidene D-glucofuranose but obtained only a very low yield, possibly due to the reaction temperature being to low. Fluorosulphonic acid was also tried as a replacement for chlorosulphonic acid with no success.

A 4% solution of sulphur trioxide gave products with indefinite composition. Grant also prepared 3,4-O-isopropylidene-6-trityl-methyl-galactoside 2-sulphate and barium lactose sulphate using pyridine sulphur trioxide complex in pyridine. Galactose 2-sulphate was obtained from the substituted galactoside 2-sulphate in very small yield by hydrolysis. The small yield may be due to desulphation during hydrolysis.

Jones et al[48] used suphuryl chloride in pyridine chloroform solution to sulphate glucose, galactose and maltose. The products obtained were the chlorosulphated sugars and cyclic sugar sulphates. Jones et al[49] have also used this route to prepare carbohydrate fluorosulphate by reacting the chloro-sulphate with silver fluoride.

Concentrated sulphuric acid has been used by Takiura et al[50] to prepare the tri-sulphates of glucose, galactose, mannose and fructose. Position of the sulphate substitution was investigated by oxidation reactions and methylation when it was concluded that aldo-hexoses reacted by positions 1, 3 and 6 and keto-hexoses reacted at positions 1, 2 and 4.

Mumma, Haiberg and Simpson[51] have shown that sulphuric acid in combination with NN dicyclohexylcarbodiimide in NN dimethylformamide solution can be used to prepare carbohydrate monosulphates in good yield.

In order to test the method they prepared 1,2,3,4-di-O-isopropylidene-α-D-galactopyranose-6-sulphate, 1,2:5,6-di-O-isopropylidene-α-D glucoguranose 3 sulphate and 2',3'-O-isopropylidene adenosine 5' sulphate in good yield. In the investigation they found that the yield was partially dependent on the order of reactant addition, on the solvent used and on the presence of water in the solvent. Product isolation was performed by separation on a DEAE cellulose column.. Fuming sulphuric acid in mixture with chlorosulphonic acid has been used[52] to prepare sorbitol sulphate by reaction in NN dimethyl-formamide admixture.

Yoshihiro Nitta et al[53] prepared polysulphates of glucuronic acid by reaction with sulphur trioxide in sulphur dioxide solution.

Infra red spectroscopy of carbohydrate sulphates

Infra red spectroscopy can be used in the elucidation of the structure of carbohydrates. A large number of carbohydrates and carbohydrate derivative spectra are listed by Kuhn[54]. Anomeric and isomeric forms of glycosides, sugars and sugar alcohols were readily distinguishable.

The sulphate group of sulphated carbohydrates gives a characteristic absorption band in the region 1230-1255 cm^{-1} due to S=O stretching vibrations and absorption bands in the 'fingerprint' region (700-1000 cm^{-1}). Orr[9] characterised the band appearing at 820 cm^{-1} in the infra red spectrum of polysulphated hyaluronic acid as being due to the C-O-S vibration of an equatorial substituted sulphate group. Comparison of the spectra of the chondroitin sulphates A and C in the finger-

print region (A: 928, 852, 725 cm^{-1}, C: 1000, 820, 775 cm^{-1}) indicated that the sulphate groups in chondroitin sulphate C are equatorially substituted. Since by chemical evidence chondroitin sulphates A and C contain the same disaccharide repeating unit; i.e. formation of chondroisine on acidic hydrolysis, the spectral differences are not due to the glucuronidic linkage but to the sulphate group. The sulphate group in chondroitin sulphate A were therefore assigned to an axial position. These results are confirmed by reference to the spectra of chondroitin sulphate B (928, 855, 712 cm^{-1}) which has the sulphate group substituted in the axial position, determined by methylation studies. Hoffman, Linker and Meyer[10] confirmed the results of Orr and Mathews with their work on the action of testicular hyaluronidase on chondroition sulphates A and C

Lloyd and Dodgson[43,55,56] prepared and examined samples of D-glucose 6-sulphate, D-glucose 3-sulphate, galactose 6-sulphate and N-acetyl-D-glucosamine 6-sulphate. It was found that substitution of a sulphate group on the equatorial primary hydroxyl group had a C-O-S stretching frequency of 820 cm^{-1}. A secondary equatorial group has an C-O-S stretching frequency at 832 cm^{-1}.

Thin layer chromatography

The use of thin layer chromatography has increased very rapidly since the introduction by Stahl[57] of strict standardisation of conditions which enabled reproducible results to be obtained. It has in many cases replaced paper chromatography in the analysis of carbohydrates because of the increased speed

of separation. Bergelsen[58] et al separated lyxose, ribose and arabinose, manose mixtures in 7 to 18 hours compared to the 8 or 9 days required for paper chromatography. In many cases there is an increased sensitivity in detection, e.g. using aniline hydrogen phthalate developer, the detection limit was 20 times lower for the sugars than when paper chromatography was used. By shaping the support layer to form bands instead of spots the separation of a mixture components can be considerably improved but with some loss of speed. Practically any column packing material can be prepared in a form suitable for thin layer chromatography.

Silica gel has been found to be a very useful material for the separation of sugars because more vigorous reagents and conditions can be used for locating the separated sugars. Gee[59] separated the α and β anomers of glycosides and furanose and pyranose isomers on silica gel using 5% sulphuric acid at 110°C to develop the spots. Thin layer chromatography can be used for the preparative separation of mixtures and a good recovery of the components can be obtained. Wolfram et al[60] separated a mixture of D-galactose and D-xylose with 80% recovery. For preparative work however paper chromatography has an advantage in that much heavier loadings can be used.

Nuclear Magnetic Resonance Spectroscopy

During the course of the literature survey it became apparent that very little work on the use of nuclear magnetic resonance spectroscopy for the analysis of uronic acids in general had been published. It was therefore both necessary and desirable to obtain reference ^{1}HNMR spectra for the original

Uronic acids used for these sulphation reactions and these traces have been given for reference.

Rees and Wight[61] have recently published the 'HNMR (100MHz) spectra for galacturonic acid which they are using in a computer simulation study to investigate the conformational properties of pectins.

General account of the work carried out upon Sulphated Carbohydrates

Sulphated uronic acid residues are found in several polysaccharides which occur in both animal and plant tissues but the sulphate group is often easily removed during the initial separation of the polysaccharide and its analysis making the determination of it's location in the uronic acid unit difficult.

Little is known about the reactions of sulphated uronic acids either chemically or physiologically and it seems desirable to prepare the sulphated elementary uronic acids in reasonable quantity and to evaluate their properties.

Very little work has been done on sulphating the elementary uronic acids but the sulphation of the monosaccharides has been studied in some detail and whilst there are many unresolved problems the general methods of sulphating the monosaccharides as described in the introduction would seem to be a reasonable starting point for preparing the uronic acid sulphates.

At the time this work was carried out the use of sulphur trioxide complex, chlorosulphonic acid and sulphuryl chloride in the presence of an organic base seemed to be the most promising and were tried with glucuronic acid and galacturonic acid.

Glucuronic acid was used as the commercially available material and galacturonic acid was prepared from a grade of pectin by enzymic hydrolysis with pectinase[62]. It was intended to use mannuronic acid as a starting material for sulphation and a sample of mannuronic acid was obtained by the hydrolysis of alginic acid with hot formic acid[63]. It proved difficult however to prepare a pure sample of mannuronic acid free from lower polymers. Crystalline material was formed after seeding and standing at 5°C. Seed crystals were obtained from impure reaction product by separation on a formate buffered ion exchange column.

Commercially available Pectin and Alginic acid were also treated with a sulphating agent (SO_2Cl_2).

Sulphation with pyridine sulphur trioxide complex

An alternative sulphation system to the chlorosulphonic acid pyridine reaction is to use pyridine sulphur trioxide complex in pyridine or chloroform. The system has some advantages over chlorosulphonic acid-pyridine system in that

it allows more vigorous reaction conditions to be used. Glucuronic acid and galacturonic acid were used in a series of reactions with this reagent in an attempt to prepare their sulphates. The uronic acid, dispersed in chloroform was reacted with pyridine-sulphur trioxide complex for different periods of time at various reaction temperatures. The reaction mixture, after treatment with dilute sulphuric acid, was phase separated into a chloroform layer and an aqueous layer. The chloroform layer, after work up, contained a very small quantity (approximately 0.5 ml) of a red-brown liquid. The aqueous layer was neutralised with barium carbonate and worked up in the presence of barium carbonate. The residue was extracted with water to obtain e product free from pyridine salts.

An alternative recovery procedure was to neutralise the solution with barium hydroxide until a pH of 9 was attained to decompose any pyridine salts present. The excess alkali was removed by treatment with carbon dioxide and filtration and the residue obtained by evaporation was extracted with water to give the product.

It was found that at temperatures less than 35°C and for time periods less than one day very little reaction occurred. At higher temperatures a mixture of products was obtained as shown by T.L.C. and paper chromatography which showed the presence of compounds in addition to the starting uronic acid. Infra-red analysis on the product mixtures in the 'fingerprint' region showed absorption bands at 1260 cm^{-1} and 850-820 cm^{-1} consistent with the presence of sulphate groups. The sulphur

content of the products were in the range 2 - 8%. It was not possible to obtain a yield of the sulphated product since no means of separating the pure uronic acid sulphate could be found. Attempts were made to extract the residues with different solvents but no appreciable separation was achieved. An attempt to separate the glucuronic acid product by preparative T.L.C. was not successful, it seems that while the products could be separated analytically the contact time and subsequent work up conditions probably caused hydrolysis to occur.

Sulphation with chlorosulphonic acid reagent

The use of chlorosulphonic acid in the presence of a base such as pyridine has been used extensively for the preparation of sugar sulphates; but suffers from the disadvantage that large quantities of inorganic ions have to be removed from the product.

The standard method as used by Percival and Souter[27] is to treat the solutions with silver sulphate and hydrogen sulphide before recovering the product. This means however that large volumes of aqueous solutions have to be handled and with very easily hydrolysed material a consequent reduction in yield and contamination of the product.

A number of methods have been tried to remove the inorganic ions without increasing the loss of material by hydrolysis. One method which shows some potential is that of Coleman and Holt[64] where the product is differentially eluted from a charcoal-keiselghur column with increasing concentrations of alcohol-water mixture.

Glucuronic acid and galacturonic acid were treated with chlorosulphonic acid in pyridine-chloroform mixture at 0-5°C

by the dropwise addition of chlorosulphonic acid to a stirred dispersion of the uronic acid in 25% pyridine in chloroform mixture. The reactions were completed for various times at different temperatures then the reaction mixture was treated with cold dilute sulphuric acid, separated into two phases and individually worked up. The chloroform layer generally gave a small amount of a dark residue. The aqueous layer after neutralisation and removal of pyridine gave a residue on evaporation which was extracted with hot ethanol to obtain the product.

Products were obtained in yields of 3% to 5% with sulphur contents ranging from 2% to 3%. Increasing the reaction times and temperature beyond an optimum appears to reduce the yield. The best conditions found were to react the mixture after the initial reaction for about three days at room temperature. Examination of the product by T.L.C. showed the presence of a mixture of compounds including the original uronic acid. Most of the other spots showed bad streaking and in some cases some of the components on the chromatograph could be hydrolysis products. Infra-red analysis indicates the presence of sulphate groups, i.e. absorption bands at 1260 cm^{-1} and at 870-850 cm^{-1}. Separation of the reaction products of glucuronic acid and galacturonic acid sulphations with chlorosulphonic acid was performed using the technique of Coleman and Holt. An equal weight mixture of charcoal and keiselghur (100 mesh) was used as a four inch packed bed in a three inch diameter column. The alcohol extracts were applied to the column as solutions in water, the inorganic ions removed with

water and the product with stepped fractions of alcohol-water mixtures. Most of the unreacted uronic acid was eluted in the 5-10% ethanol fractions and the later fractions eluted with 10-20% ethanol were collected for further investigation. The recovered products (approximately 20%) would indicate that if all the material is eluted from the column than a comparatively large amount of inorganic material was present in the extraction product.

Sulphuryl chloride reagent

Sulphation of glucuronic acid and galacturonic acid with sulphuryl chloride were carried out in pyridine chloroform mixture in a manner similar to that of Jones et al[48]. This method requires the addition of the dried carbohydrate to a cold preformed mixture of sulphuryl chloride in pyridine-chloroform. After reaction the mixture was worked up by removing excess sulphating agent, neutralising and separating the product contained in the chloroform layer.

Very little product residue remained after removal of the chloroform (1 ml) and so a number of variations in sulphation conditions were tried. Changing the reaction temperature showed little improvement in yield but at reaction temperatures over 40°C the mixture became a dark brown tar indicating degradation (or polymerisation) of the carbohydrates. It was thought that impurities (e.g. S_2Cl_2) in the sulphuryl chloride might be affecting the reaction and some of these reactions were repeated using a colourless fraction of sulphuryl chloride distilling at 69°C, 760mm. Essentially the same results were obtained but since the impurity level seemed to

be high, e.g. 150-200 ml of the first fraction had to be taken off before a suitable fraction was obtained from 500 ml of commercial reagent; all subsequent runs were made with purified reagent. Modifications to the technique were used where the carbohydrate was first treated with the pyridine at a temperature less than 30°C to try and form a saturated solution before the addition of the diluent chloroform. The sulphuryl chloride was then added dropwise to the cooled reaction mixture at a rate suitable to maintain the required reaction temperature.
No improvement in yield reaction occured until the temperature was taken high enough, to cause tar formation.

During these reactions it was observed that a large amount of a white or yellow solid (5-6g) was formed which had to be filtered off. Since the carbohydrate contained a carboxyl group it seemed possible that the sulphated or chlorosulphated uronic acid might form a pyridine salt insoluble in the chloroform layer. The solid filtered off was therefore examined by elemental analysis and by then layer chromatograph y.
Elemental analysis indicated the presence of nitrogen and sulphur in suitable proportions for pyridine sulphate, e.g.

for mono-sulphate C = 16.81%, H = 3.66%, S = 11.8%

for di-sulphate C = 20.38%, H = 2.83%, S = 18.1%

for cyclic sulphate C = 28.15%, H = 3.15%, S = 12.5%

Pyridine sulphate C = 33.85%, H = 3.14%, S = 18.1% N = 4.92%

Analysis for chlorine showed the presence of 1-2% of chlorinated material.

T.L.C. results showed the presence of some material with an Rg value of 2.17 which could be sulphated uronic acid but the quantity of product was not ascertainable since the spots

could be faint due to either poor development or small quantities of product. The developed chromatograms usually showed badly streaked product spots which could be due to hydrolysis of the sulphate taking place during the analysis. The elemental analysis results indicate that only very small amounts, if any, of carbohydrate sulphate were present.

Examination of the aqueous layer from a glucuronic acid run showed, after neutralisation with barium carbonate and removal of pyridine by ion exchange resin, only the presence (T.L.C.) of some glucuronic acid. A further extension in the time of reaction to periods up to a week at room temperature did not give any improvement in results and it would seem that sulphuryl chloride does not sulphate monosaccharide uronic acids readily.

Both pectin and alginic acid were apparently insoluble in the reaction medium at all stages of the reaction and the products on analysis contained 8% - 1% sulphur and 1% - 3% chlorine indicating the possibility of some sulphation.

Problems involved in the purification of sulphated uronic acids

The separation of the components of uronic acid sulphation reactions was attempted using column chromatography with both silica gel and cellulose as stationary phase. Whilst both these absorbants could be used for the analytical separation of the reaction mixture no clean cut separation was obtained by column chromatography. The separation characteristics by thin layer chromatography should have allowed separation on columns and it is suggested that the running time of the

columns was sufficient to allow substantial hydrolysis of the sulphated products to occur. With the silica gel columns it is also thought that sufficient water is retained by the silica get, even after drying and pretreating the column with a large amount of the eluant, to change the eluant characteristics. Preparative thin layer chromatography using 1 mm thick layers of silica gel was attempted but with the run time needed for this layer thickness the components formed a diffuse band. Arbitary separation of this band showed (T.L.C.) a mixture of compounds in each fraction suggesting that some decomposition of the product had occurred.

In the sulphation reactions it was found that the pyridine base used caused considerable trouble in the later stages of work up. Pyridine sulphate is not completely decomposed with barium carbonate and it requires either treatment by barium hydroxide (pH 9) or repeated evaporation with barium carbonate to remove the pyridine salts without the formation of a strong acid solution.

It was found to be advantageous in the work up of these products to treat the neutralised solutions with a cationic exchange resin to remove pyridine from the solution. For small scale work an ion exchange column was most convenient but for larger scale work a preliminary treatment of the solution with ion exchange resin followed by passage through a column of the resin was better.

The major problems encountered were low reaction yields and difficulty in purification. The most satisfactory sulphating agents examined were chlorosulphonic acid in pyridine and pyridine-sulphur trioxide complex in chloroform. The low

reaction yields were probably due to poor reaction rates due to low solubility in the reaction medium. It has been shown by Turvey et al[41] that an equilibrium between uronic acid and sulphate is also a limiting condition in the reaction yield of saccharide sulphation. If an equilibrium reaction is attained then a low solubility of the uronic acid in the medium used would tend to be limiting on the rate at which the reaction proceeds. If a better solvent for the uronic acid is used as reaction medium then the danger of increased side reactions such as epimerisation or oxidation become more important.

Two problems occur in purification: (1) unreacted starting material appears to track with the reaction product, (2) the possibility that hydrolysis occurs very rapidly. Hydrolysis complicates the problem since it is difficult to ascertain if the starting material found at all stages is due to hydrolysis or non separation of the uronic acid from the sulphate. An improvement in the solubility of the product in organic solvents, e.g. by formation of the methyl ester[8] could enable the purification to be carried out in anhydrous conditions. In addition this could introduce sufficient difference in physical properties to allow them to be separated. The methyl esters however would probably be unstable and require careful handling.

Results and Discussion

A number of preparative routes for uronic acid sulphates have been investigated in an attempt to find a convenient preparative method.

With all of the sulphating agents used for the sulphation of glucuronic and galacturonic acids only small quantities of a sulphated product were detected. Estimation of the amount of uronic acid sulphate formed had to be based on the sulphur content of the crude product because no satisfactory method of purification could be found.

Pyridine-sulphur trioxide complex in chloroform gave products with sulphur values of 1% to 4%. Infra-red and T.L.C. analyses indicated the presence of a sulphate group and a compound with an M_{gu} value of 1.6 - 1.7. Increase of reaction time and/or temperature did not materially increase the apparent yield.

Chlorosulphonic acid reagent in the presence of pyridine base gave products with sulphur values of 1% to 13%. Infra-red analysis showed the presence of a sulphate group (bands at 1260 cm^{-1}, 870-820 cm^{-1}, Figure 2&4) and innophoresis showed the presence of a compound with an M_{gu} = 1.6 - 1.7. Increase of the time and/or temperature of reaction did not appear to materially increase the apparent yield of sulphated product but ultimately resulted in the formation of a tar.

Sulphuryl chloride reagent in the presence of pyridine base appeared to give only very small amounts of a sulphated reaction product with either glucuronic acid or galacturonic acid. Only the salt formed in the reaction contained any product with a sulphur value above 1%. T.L.C. examination of the salt showed the possibility of the presence of a very small amounts of a reaction product. The reaction mixture after salt removal

extraction and concentration gave a syrup containing less than 1% sulphur. Chlorine analysis however was always positive suggesting that some chlorination had occurred, probably the formation of an uronic acid chlorosulphate.

T.L.C. gave no clear indication of the presence of reaction products; the chromatograms always showed badly streaked spots. An increase of time and/or temperature of reaction gave tars.

Alginic and pectic acids were also treated with this reagent. In these cases the starting material was never appreciably soluble in the reaction mixture and the solids left after reaction could be separated by filtration and washing with methanol. Analysis of the insoluble residue showed sulphur contents from 0% to 8% and chlorine values up to 3%. Infra-red analysis showed the presence of a sulphate group (1230 cm^{-1}, 820 cm^{-1}) for alginic acid and at 1230 cm^{-1} and 810 cm^{-1} for pectic acid. This suggests that sulphation of the uronic acid does take place but that it is the chlorosulphate which is formed.

These reagents are known to be effective for the sulphation of monosaccharides and it seems unlikely that the presence of a carboxyl group would, in itself, inhibit the sulphation reaction. A number of possible explanations for a low yield can be advanced:

1. the sulphated uronic acid is formed but is unstable and is decomposed by the work up.
2. the reactant concentrations are very low reducing the reaction rate.
3. an equilibrium state is set up in which the overall equilibrium does not favour the sulphated product.

Purification of the crude reaction product proved to be one of the major difficulties in attempting to prepare the sulphated uronic acids. Inorganic ions could be removed by extraction and separation on a charcoal column. The reaction products were then extracted from the charcoal by gradient elution with ethanol-water mixture but no appreciable separation of the product was obtained, Attempts to purify these products by cellulose column chromatography with eluant B did not give any distinct separations although changes in concentration of the components was observed in the fractions.

A silica gel column packing was not satisfactory since the column packing always contained sufficient water to change the eluant composition. Analytical thin layer chromatography was satisfactory for detecting reaction components but preparative T.L.C. under the same conditions was not successful, only a diffuse band of material was obtained.

Extraction of the layer holding this band indicated a diminished amount of the presumed sulphate which might indicate a slow hydrolysis of the uronic acid sulphate.

To explain the poor separation it is suggested that the acid nature of the uronic acid causes the reaction product to track with the starting material. With adsorption on active charcoal columns it is reasonable to assume that the charge binding the uronic acid to the active carbon sites is sufficiently similar to prevent distinct desorption.

For partition separation on cellulose columns the separation of starting material and reaction product will depend on their relative solubilities and concentrations in the two phases. With free uronic and mon-sulphated uronic acid it is

probable that the solubilities of the two components in the system used are sufficiently similar that separation will only occur if the resolving power of the system is large (e.g. by T.L.C.).

Ion exchange systems will also suffer from the same type of problem and require a very delicate balance of eluting conditions to effect any separation. In addition any separation based on solutions of the materials will necessitate using polar solvents when the sulphate groups of carbohydrates tend to become sensitive to exchange reactions so that prolonged or vigorous exposure to these conditions will tend to decompose the reaction product with the formation of more strongly acidic conditions.

Separation of the sulphated reaction products might be helped by blocking the functional groups of the uronic acid by ca. esterification when the stability and physical properties pf the sulphated product could be better controlled. This would however involve the preparation of uronic acid derivatives with unknown stabilities and the removal of the blocking groups in the presence of what is probably a very labile sulphate group could also lead to difficulties.

Infra-red analyses of the reaction products were of course confused by the presence of impurities but some indication of carbohydrate sulphate was found by diffuse bands in the region 1250-1220 cm^{-1} and bands in the 1000-700 cm^{-1}. region. It has been reported that absorption bands appearing at 1000, 820 and 775 cm^{-1} are due to the C-O-S stretching frequency of an equatorial substituted sulphate group and similarly bands at 928, 852 and 725 cm^{-1} indicate an axial sulphate. Although there is some variability in the position of the bands appearing

between 850-800 cm^{-1} in the spectra of the uronic acid sulphates reported here the presence of an absorption at 790-770 cm^{-1} suggests that the sulphates are equatorial indicating that substitution is in position C2, C3 or C4 with glucuronic acid and in position C2 or C3 with galacturonic acid.

With sulphuryl chloride reagent only small traces of a sulphated uronic acid product were detected.

A pure sample of mannuronic acid was prepared from alginic acid by hydrolysis with hot 88% formic acid. Difficulty in obtaining crystalline material from a syrup made it necessary to use a 'seed' prepared by the separation of a small amount of uronic acid on a formate buffered anionic exchange resin (Permutite SRA 101) with formic acid eluant. The separation was not clean but did give a satisfactory 'seed' material.

A pure sample of galacturonic acid was prepared from pectin by enzymic hydrolysis with pectinase. The product was purified by separation on a cationic exchange resin.

Glucuronic acid, galacturonic acid and mannuronic acid were treated with dueterium oxide to reduce the amount of hydroxyl proton signal by D-H exchange and NMR spectra obtained. The spectra are reproduced here for comparison purposes. NMR spectra of some of the products are also given. It was intended to try and obtain data about the effect of a sulphate group in the uronic acid on the NMR spectra by spin-spin decoupling but it was not possible to obtain the data in time.

It is suggested that future work to achieve a preparative route for uronic acid sulphate might be to use a more polar

solvent such as dimethylformamide or dimethylsulphoxide to increase the reactant concentration and therefore to increase the effective rate of reaction. This could however lead to other difficulties such as the formation of di and tri sulphates, inversion and anhydro ring formation. In addition under these conditions the substituted sulphate group would be subjected to exchange reactions with the system by-products. If the equilibrium conditions noted by Turvey et al for the sulphation of monosaccharides also applies to the formation of uronic acid sulphates it would be very desirable to try and drive the equilibrium towards the sulphate by the removal of the sulphate from solution.

To minimise these side effects it would be advantageous to block the groups of the uronic acid not intended for reaction. This however would involve several more reactions of poorly defined products and it would be better to prepare the monosaccharide derivatives, possibly sulphate these, then obtain the uronic acid sulphate by oxidation of the primary hydroxyl to a carbonyl group and removal of the blocking groups. A major difficulty with this method would be the sensitivity of the sulphate group to oxidising conditions. This might be overcome by methylating the sulphate group before oxidation and using a neutral oxidising system such as palladium-oxygen.

As a first step however the use of the sulphuric acid cyclohexyl-carbodiimide reagent should be investigated as a sulphating agent for uronic acids.

EXPERIMENTAL

Glucuronic acid and galacturonic acid were reacted with pyridine sulphur trioxide complex, chlorosulphonic acid and sulphuryl chloride over a range of conditions to assess the best conditions for preparing uronic acid sulphates.

Reagents

Pyridine sulphur trioxide complex was prepared by the method of Guiseley and Ruoff[45].

Chlorosulphonic acid was used as supplied.

Commercial sulphuryl chloride was purified by distillation over copper gauze packing in a short fractionating column, the fraction b_{760}, 68.5-69.5°C being taken.

Pyridine was dried and purified by refluxing over KOH and then distillation, b_{760} 112-114°C.

Chloroform (A.R.) was dried by standing over anhydrous sodium sulphate and a freshly distilled fraction b_{760} 59.5-60.5°C used for each run.

Thin layer chromatography

The glass chromatography plates were cleaned with alcoholic KOH, rinsed with water, washed with dilute acetic acid, washed with distilled water and polished with a lint free cloth before coating.

Chromatograms were run using an 0.25mm layer of silica gel or cellulose spread from aqueous suspension. The plates were preheated at 110°C for 15 minutes before use.

Samples were applied, using capilliary pipettes, as spots of approximately 1% solutions in water or alcohol. In general three applications were made for each spot.

After running the chromatogram was dried at 110°C and sprayed with a developing reagent then development completed by heating at 110°C for 3 to 10 minutes.

The eluants used were: -

(A) Neutral solvent

butan-1-ol	40 parts
ethanol	10 parts
water	50 parts

(B) Acid solvent

Butan-1-ol	40 parts
acetic acid	10 parts
water	50 parts

(C)	ethyl acetate	18 parts
	formic acid	1 part
	acetic acid	3 parts
	water	1 part

Eluants Aand B were prepared for use by equilibriating at reflux for 1 hour, cooling to room temperature and standing for 1 day then removing the lower aqueous layer. A further 2 parts by volume of butanol was then added to the upper alcohol layer.

Developing reagents:

(I) Aniline hydrogen phthalate

0.93g aniline + 1.66g phthalic acid in 100 ml of water saturated butanol

(J) Benzidine trichloroacetic acid

1g benzidine in 20ml acetic acid,

8g trichloroacetic acid in 20ml water,

combined and made up to 200ml with ethanol.

(K) Naphthoresorcinol-sulphuric acid

0.1g naphthoresorcinol in 100ml ethanol

10% H_2SO_4 mix equal parts just before use.

(L) Naphthoresorcinol-phorphoric acid

0.1g naphthresorcinol in 100ml ethanol,

six 1 ml naphthoresorcinol solution +

1 ml H_3PO_4 with 18 parts ethanol just before use.

Paper chromatography

Chromatograms were run on Whatman No. 1 filter paper. Samples were applied as spots of approximately 1% solutions in water or ethanol using capilliary pipettes.

Chromatograms were usually run for two days, dried, sprayed with a developing reagent and heated in an oven at 110°C for 5 to 15 minutes.

Reference materials were glucose in 50/50 ethanol-water or glucuronic acid in aqueous Sodium bicarbonate solution.

Eluants and developing reagents were the same as for thin layer chromatography.

Ionophoresis

Separations were carried out with Whatman No.1 paper in phosphate or acetate buffer. Samples were applied to the paper support as spots of approximately 1% solutions in water

or ethanol with capilliary pipettes. After applying the samples the test paper was saturated with buffer, sandwiched between glass plates and placed in a Shandon Horizontal innophoresis tank. A voltage was then applied to the circuit and varied over a period of time so that the current drawn remained constant. The test paper was then dried at 110°C, sprayed with developing reagent and development completed by heating for 5 to 10 minutes at 110°C.

Buffers:-

Phosphate pH10

8.9g disodium hydrogen phosphate + 0.33g sodium hydroxide dissolved in 1 litre of water.

Acetate pH4

0.2N Acetic acid + 0.2N sodium acetate.

Developers:- as for thin layer chromatography.

Preparation of samples for NMR analysis

The carbohydrate sample to be examined was treated with deuterium oxide to prevent hydroxylic proton interference. The sample (0.1g - 0.3g) was repeatedly (5 times) dissolved in D_2O (3ml) and evaporated to dryness under reduced pressure. After this treatment the sample dissolved in D_2O was run on a Perkin Elmer R10 machine using tetra methyl silane as reference standard.

Infra-red Analysis

Sample spectra were run on a Perkin Elmer 237 Infra red Spectrometer as a 2-3% mixture of the sample in KBr discs, the spectra between 4000 cm^{-1} and 600 cm^{-1} being obtained.

Reaction with pyridine sulphur trioxide complex

The reaction was carried out in a dry 250ml 3 neck flask fitted with a stirrer and silica gel guard tube.

Glucuronic acid (4g), dired in vacuo over P_2O_5, was dispersed in freshly distilled pyridine (50ml) then pyridine sulphur trioxide complex (6.5g) added to the stirred mixture. The reaction was then continued at the required temperature for a specified time. When the reaction was complete the mixture was poured into water (200 ml), neutralised with barium carbonate, filtered and evaporated under reduced pressure (45°C, 15mm) in the presence of barium carbonate to small volume. The suspension was diluted to 50ml, filtered and evaporated to dryness. The residue was extracted with 85% ethanol in an attempt to purify the product.

Reaction with chlorosulphonic acid

The reaction was carried out in a dry 250ml 3 neck flask fitted with a stirrer and a silica gel guard tube, low temperature thermometer and dropping funnel. Dry glucuronic acid powder (4g) was added to a mixture of dry chloroform (100ml) and pyridine (30ml). The resulting suspension was cooled to -5°C and 6ml of chlorosulphonic acid in 10ml chloroform added dropwise to the reaction mixture. The addition of chlorosulphonic acid was stopped when necessary to prevent the temperature exceeding 0°C. when the addition of the chlorosulphonic acid was complete stirring was stopped and the reaction mixture allowed to rise to room temperature over night. The mixture was reacted for periods of time at varying temperatures. After reaction the mixture was poured into ice

cold dilute sulphuric acid, neutralised with barium carbonate, filtered and the organic layer separated. The organic layer was washed with dilute sulphuric acid, water, sodium bicarbonate solution, water, dried over calcium chloride and evaporated to dryness (45°C, 15mm). The aqueous layer was evaporated to 50ml (45°C, 15mm) in the presence of a small amount of barium carbonate, filtered and passed down a column of cation exchange resin, ZeoCarb 225. The eluant was neutralised with barium carbonate, filtered, evaporated to 50ml and passed down a charcoal-keiselghur column. The column was washed with water until the chloride ion was removed ($AgNO_3$) then with 5%, 10%, 15% ethanol in water. The alcohol eluate was evaporated (45°C, 15mm) to dryness.

Reaction with sulphuryl chloride

The reactions were carried out in a dry 250ml 3 neck flask fitted with a stirrer and a silica gel guard tube. Dry glucuronic acid powder (4g) was dispersed in a mixture of dry chloroform (100ml) and pyridine (30ml). The suspension was cooled to 0°C and 7ml of sulphuryl chloride added dropwise. The addition of sulphuryl chloride was stopped as necessary to keep the temperature of the mixture below 10°C. When the addition of the sulphuryl chbride was complete the temperature of the mixture was allowed to rise and the mixture reacted at different temperatures for varying times. After reaction the mixture was poured on to ice cold dilute sulphuric acid, neutralised with barium carbonate, filtered and the organic layer separated. The organic layer was washed with dilute sulphuric acid, water, sodium bicarbonate solution, water,

dried over anhydrous calcium chloride then evaporated to dryness (45°C, 15mm). The aqueous layer was evaporated to dryness in the presence of barium carbonate. The residue was extracted with hot 85% ethanol, water and the extracts evaporated to dryness.

Sulphation of uronic acids with pyridine-sulphur trioxide complex

Glucuronic acid (4g) was reacted with $Py.SO_3$ (6.6g) in pyridine (50ml) for 2 hours at 30°C. The reaction mixture was poured into ice-water mixture (200ml), stirred until the ice had dissolved (approximately 20 min.). Barium carbonate (10g) was then added in small portions to the stirred reaction mixture until effervescence had virtually ceased. The solution was then filtered using a Buchner filter apparatus and the filtrate pH value measured as 7 to 8 using BDH universal indicator paper. The filtrate was then concentrated under reduced pressure using a rotary evaporator. To prevent the formation of acidic conditions during concentration the solution was tested periodically for pH value and neutralised as necessary with $BaCO_3$. The final concentration to dryness was carried out in the presence of barium carbonate (approximately 4g). The concentrate was then extracted with petroleum ether (40-60), ethyl acetate, dioxan, ethanol and water. After filtration each extract was evaporated to dryness. Only the ethyl acetate and ethanol extracts gave any indication of a residue, in both this was a faint smear on the walls of the evaporation flask.

Glucuronic acid (4g) was reacted with $PySO_3$ (6.55g) in pyridine (50ml) for 2 days at 30°C and worked up as previously described. The concentrate was extracted with ethyl acetate, ethanol, petroleum ether (40-60), dioxan and water. The ethanol extract gave a small amount of a transparent material.

Glucuronic acid (4g) was reacted with $PySO_3$ (6.6g) in pyridine (50ml) for 2 days at 60°C. After work up and

concentration to dryness as before the product was extracted with water, filtered and the neutral filtrate evaporated to dryness. A yellow powder (3.5g) was obtained which had a sulphur content of 4.15%. The theoretical analysis for glucuronic acid sulphate should be C = 17.55%, H = 2.153%, S = 7.8%, O = 39.05%, Ba = 33.4% for the barium salt or C = 26.3%, H = 3.66%, S = 11.7%, O = 58.45% for the free acid.

Ionophoresis in phosphate buffer showed the presence of two compounds in addition to glucuronic acid with M_{gu} = -0.04 and 1.7. Thin layer chromatography using neutral solvent (A) also showed the presence of two compounds in addition to glucuronic acid with R_g = 0.244 and 1.9. Infra red analysis showed the presence of absorption bands at 1260-1225 and 845 cm^{-1}. Purification of this reaction product was attempted by cellulose column chromatography using solvent (A). Separation did not take place as with normal partition chromatography. A stationary yellow band was formed at the top of the column and also a yellow coloured mobile band. The column was eluted and fractions collected. The column was then unpacked and separated. The fractions obtained were: F1 packing holding adsorbed material, F2 remainder of packing, F3 eluant containing mobile band, F4 eluant collected up to the mobile band. Fractions F1 and F2 were recovered from the cellulose packing by extraction with water (3 x 100ml). All the fractions were evaporated to dryness and dried under reduced pressure over P2O5. T.L.C. with solvent (A) showed that all fractions except F4 contained a slow moving compound with R_g = 0.4. Fractions F2, F3 and F4 contained a compound

with R_g = 1.92. Ionophoresis showed that F1 and F2 contained glucuronic acid and a compound with M_{gu} = 1.74. Fraction F2 also showed the presence of a compound with M_{gu} = 0.074.

Glucuronic acid (4g) was reacted with $PySO_3$ (6.5g) in pyridine (50ml) for 4 days at 30°C as previously described. The product was extracted with ethanol (2 x 30ml portions) and 0.4g of a pale yellow gum obtained having a sulphur content of 2.24%. Infra red spectrum analysis showed adsorption bands at 1250)1220 cm^{-1} and at 825 cm^{-1}.

Glucuronic acid (4g) was reacted with $PySO_3$ (6.55g) in pyridine (50ml) for 4 days at 40°C as previously described. The reaction product was extracted with ethanol (2 x 30ml) and the extract evaporated to dryness. The extract was redissolved in ethanol (50ml) and concentrated under reduced pressure until a yellow precipitate formed (approximately 15ml of ethanol remaining). This precipitate was filtered off and dried to give 0.2g of a product with a sulphur content of 8.24%

Ionophoresis using phosphate buffer showed the presence of a compound with M_{gu} = 1.655. Infra-red analysis showed the presence of adsorption bands at 1255 cm^{-1}, 840 cm^{-1} and 720 cm^{-1} (IRT 3).

Galacturonic acid (4g) was reacted with $PySO_3$ (6.6g) in pyridine (50ml) for 2 days at 30°C as described for glucuronic acid. The reaction product was extracted with petroleum ether (40-60), ethyl acetate, dioxan, ethanol and water. The ethanol extract (2 x 50ml) gave 0.1g of a transparent gum which had a sulphur content of 2.8%.

Galacturonic acid (4g) was reacted with $PySO_3$ (6.5g) in pyridine (50ml) for 2 days at 60°C as previously described. The product was extracted with water filtered and concentrated under reduced pressure to give a yellow powder (3g) which had a sulphur content of 2.25%. Ionophoresis in phosphate buffer indicated the presence of galacturonic acid M_{gu} = 0.858 and a compound with M_{gu} = 1.58 but other compounds could have been present as indicated by severe 'streaking'.

Galacturonic acid (4g) was reacted with $PySO_3$ (6.5g) in pyridine (50ml) for 4 days at 30°C as described. The reaction product was extracted with ethanol (2 x 30ml) to give 0.5g of a pale yellow gum which had a sulphur content of 2.44%. Infra red analysis showed adsorption bands at 1270-1250 cm^{-1}, 820 cm^{-1} and 744 cm^{-1} (IRT 1).

Galacturonic acid (4g) was reacted with $PySO_3$ (6.3g) in pyridine (50ml) for 4 days at 40°C. The reaction product was extracted with ethanol (2 x 35ml) to give 0.8g of a yellow gum which had a sulphur content of 2.84%. This gum was redissolved in ethanol and slowly concentrated but no precipitation was obtained. Attempts to obtain a solid precipitate by the addition of portions of petroleum ether (40-60) were not successful, in every case an oil and ultimately a gum was obtained.

Sulphation of uronic acids with Chlorosulphonic acid

Glucuronic acid (2g) in pyridine (30ml) was cooled under anhydrous conditions in a solid CO_2/alcohol bath to a temperature of -15°C. Chlorosulphonic acid (4ml) was added

dropwise to the stirred reaction mixture with the rate of addition being adjusted so that the reaction temperature was maintained between -15°C and -5°C. When the reagent addition was complete stirring was continued for 1 hour at -15°C then the reaction was allowed to stand for 24 hours during which time the temperature increased to ambient. The reaction mixture was then poured into an ice-water mixture (200ml), stirred for 10 minutes then neutralised with $BaCO_3$ (10g) added in portions. The mixture was filtered and evaporated to dryness, neutral conditions being maintained by treatment with $BaCO_3$ as necessary with the final concentration being carried out in the presence of $BaCO_3$. This reaction product was extracted with water (2 x 30ml), filtered and the filtrate passed down a 4" by 3" diameter charcoal column prepared as previously described. The column was eluted with water until all the $BaCl_2$ had been removed as indicated by the absence of chloride ions ($AgNO_3$ test) in the eluate. The column was then eluted with 200ml portions of 5%, 10%, 15%, 20% and 25% v/v ethanol in water. The 5% fraction on evaporation gave approximately 0.03g of a transparent gum which ionophoresis indicated was glucuronic acid. The 15% and 20% fractions after concentration under reduced pressure to low volume (20ml) were shown by ionophoresis in phosphate buffer to be the same material both having a compound with M_{gu} = 1.72 and some glucuorinc acid. These two fractions were combined and evaporated to dryness to give 0.06 g of a yellow gum having a sulphur content of 8.34%. Infra red analysis showed adsorption bands at 1250-1210 cm^{-1}, 820 cm^{-1} and 775 cm^{-1} (IRT 4).

Galacturonic acid (4g) was reacted with $ClSO_3H$ (6ml) in pyridine (30ml), as described for glucuronic acid. The product was purified by ethanol/water elution from a charcoal column, the 15% and 20% fractions giving 0.1g of a yellow gum having a sulphur content of 12.88%. Ionophoresis in phosphate buffer showed the presence of two compounds with M_{gu} = 0.858 and 1.59. Infra red analysis showed adsorption bands at 1230 cm^{-1} and 870-850 cm^{-1}.

Glucuronic acid (4g) in pyridine (30ml) and chloroform (100ml) was cooled under anhydrous conditions to -5°C in a solid CO_2/alcohol bath. Chlorosulphonic acid (6ml) was added dropwise to the stirred reaction mixture at such a rate that the reaction temperature was maintained between -5°C and 0°C. When the addition was complete stirring was continued for 1 hour at 0°C. The reaction was then allowed to stand for 2 days at ambient temperature. The reaction mixture was then poured on to ice cold 10% H_2SO_4 (200ml) and stirred to thoroughly mix the acid and reaction mixture, then the mixture was transferred to a separating funnel and the chloroform layer run off. The aqueous layer was neutralised with $BaCO_3$ (BDH universal pH paper), filtered and concentrated in the presence of $BaCO_3$ (10g). to approximately 50ml, filtered and the filtrate passed down a cation exchange column, ZeoCarb 225, to remove residual pyridine. The eluate was neutralised with Ba Carbonate, filtered, concentrated to 50ml and passed down a charcoal column as previously described. Only the 5% ethanol fraction contained any carbohydrate giving 0.4g glucuronic acid. The chloroform portion of the reaction product was washed with dilute H_2SO_4(20ml)

water (2 x 20ml), saturated $NaHCO_3$ solution (20ml) and water (20 ml) dried over $CaCl_2$ (anhyd) and evaporated under reduced pressure to dryness giving approximately 0.25ml of a brown liquid.

Glucuronic acid (4g) in pyridine (30ml) chloroform (100ml) mixture was reacted with $ClSO_3H$ (6ml) at -5°C to 0°C and at ambient temperature for 4 days as previously described. The neutralised product from the ion exchange column was concentrated to dryness in the presence of some $BaCO_3$ and the residue extracted with warm (approximately 60°C) 85% v/v ethanol. The extract was then concentrated to dryness and 1g of the residue in 50ml of water was fractionated on a charcoal column as described previously. The 15% - 25% combined ethanol fractions gave 0.2g of a yellow gum having a sulphur content of 2-15%. The chloroform portion of the reaction mixture gave only a slight smear of a brownish liquid after work up and removal of the chloroform.

Galacturonic acid (4g) in pyridine (30ml) and chloroform (100ml) was reacted with $ClSO_3H$ (6ml) at -5 to 0°C and for 2 days at ambient temperature and the product processed as described for glucuronic acid. The chloroform portion of the reaction mixture gave small quantity (0.5ml) of a brown liquid. The aqueous portion of the reaction mixture gave 0.2g of product in the 5% ethanol fraction from the separation on a charcoal column. This product was shown to be galacturonic acid by ionophoresis in phosphate buffer.

Galacturonic acid (4g) in pyridine (30ml) and chloroform (100ml) was reacted with $ClSO_3H$ (6ml) at -5°C as previously described and then for 2 days at 40°C. The reaction mixture

was processed as for previous examples. 0.5g of the ethanol (85%) extract was fractionated on a charcoal column with fractions (300ml) at 7.5%, 15%, 20% v/v of ethanol in water. The fraction at 7.5% contained 0.03g of product with a sulphur content of 0% and the 15% ethanol fraction gave 0.07g of a yellow gum having a sulphur content of 2.74%. Infra red analysis of the 15% fraction showed adsorption bands at 1250 cm^{-1} and 815 cm^{-1}.

Galacturonic acid (4g) in pyridine (30ml) and chloroform (100 ml) was reacted with $ClSO_3H$ (6ml) at -5°C and then for 2 days at 50°C and processed as described in the previous example. The 7.5% ethanol fraction (0.03g) had no sulphur and the 15% ethanol fraction (0.09g) contained 2.75% sulphur. Infra red analysis of the 15% fraction showed adsorption bands at 1250-1220 cm^{-1} and 850 cm^{-1} (IRT 24).

Sulphation of uronic acids with sulphuryl chloride

Distilled sulphuryl chloride (6.5ml) was added dropwise under anhydrous conditions to a cold (0°C) solution of pyridine (30ml) in chloroform (100ml) at such a rate that the temperature was maintained between 0°C and 10°C. When the reaction was complete the mixture was cooled to -70°C in a solid CO_2/alcohol bath and glucuronic acid (4g) added in portions (approximately 1g) and the mixture shaken to disperse the uronic acid. After the addition was complete the mixture was left in the cold bath without the addition of any more refrigerant for 1 day. The reaction mixture was then poured on to an ice-water mixture (200ml) and stirred for 10 minutes. A copious white precipitate

was formed which was filtered off. The filtrate was transferred to a separating funnel and the chloroform layer run off, washed with 10% H_2SO_4 (20ml), water (2 x 50ml), saturated $NaHCO_3$ (30ml) and water (50ml). The chloroform solution was then dried over anhydrous $CaCl_2$, and concentrated to dryness under reduced pressure to give approcimately 1.0ml of a red-brown liquid. T.L.C. with solvent (B) and developer I showed a streaked chromatogram with front R_g = 2.8 and tail R_g = 0.6 with no definite compound present but indications of the presence of a compound with R_g = 2. The product was set aside to see if crystallisation would occur but none formed over a period of 2½ years.

Glucuronic acid (4g) in pyridine (30ml) and chloroform (100ml) was cooled to -5°C under anhydrous conditions. Sulphuryl chloride (7ml) was added dropwise to the stirred mixture at such a rate that the reaction temperature was maintained at -5°C to 5°C. When the addition was complete the rea ction was continued at 35°C for 2 hours. The reaction mixture was then poured on to an ice-water mixture (200ml) and stirred for 10 minutes. The crystalline precipitate which formed was filtered off and the chloroform layer separated from the filtrate and processed as previously described. Approximately 1 ml of a red-brown liquid was obtained from the chloroform phase. The crystalline product (approximately 2g) contained 18.55% S and 0.42% Cl. The product was dissolved in methanol (50ml) then evaporated (45°C, 15mm) to a syrup which contained crystalline material. After standing the crystals were separated by pressing between filter paper.

After recrystallisation from acetone the crystals analysed for S = 16.79%. T.L.C. with solvent (B) with R_g = 2.165 which had a pink colour when developed with spray I. The syrup was extracted from the filter paper with methanol and evaporated to dryness. T.L.C. with solvent (B) and developer (I) showed three compounds with R_g = 0.445, 0.78, 2.165, all the spots when developed had a pink colour. Infra red analysis showed indications of absorption bands at 1240-1200 and 870-840 cm^{-1}.

Glucuronic acid (4g) was treated with SO_2CL_2 (7ml) at 0°C in pyridine (3oml) chloroform (100ml) mixture until all the acid chloride had reacted and then reacted for a further two hours at 35°C. A crystalline product (2g) was obtained by filtration of the mixture. A portion of the product was extracted with T.H.F. (100ml) and the solution evaporated under reduced pressure to dryness. The residue was also dried under reduced pressure.

Analysis gave:-

T.H.F. soluble: S = 18.02%. I.R. showed a broad diffuse trace with indications of absorption bands at 1250-1220 and 850 cm^{-1}.

T.H.F. insoluble:- S = 11.35%. I.R. showed absorption bands at 1240-1220 and 875 cm^{-1}.

Glucuronic acid (4g) was reacted with SO_2Cl_2 (6.5ml) in pyridine (30ml) chloroform (100ml) mixture for two hours at 50°C after the initial reaction was over. The product was a black tar.

Glucuronic acid (4g) was treated with SO_2Cl_2 (7ml) in pyridine (30ml) chloroform (100ml) mixture for two hours at 25°C after the initial reaction was over to give a white crystalline product which contained 20.45% sulphur.

Galacturonic acid (4g) was treated with SO_2Cl_2 (8ml) in pyridine (3oml) chloroform (100ml) mixture for two hours at 35°C after the initial reaction was over to yield a crystalline product containing 17.66% sulphur.

Galacturonic acid (4g) was treated with SO_2Cl_2 (6.5ml) in pyridine (30ml) chloroform (100ml) mixture for two days at 25°C after the initial reaction was over to give a crystalline product containing 17.99% sulphur.

Galacturonic acid (4g) was treated with SO_2Cl_2 (7ml) in pyridine (30ml) chloroform (100ml) mixture for 6 hours at 30°C after the initial reaction was over to yield a crystalline product with analysis S = 18.24%, C = 37.69%, H = 4.88% N = 7.4%. T.L.C. on cellulose using solvent (D) with developer (L) showed a gray streak with front having R_g = 3.75 and tail R_g = 1.25. T.L.C. using solvent (D) and developer (L) gave a pink streak with front R_g = 2.4 and tail R_g = 1.3.

Glucuronic acid (4g) was treated with SO_2Cl_2 (12.5ml) in pyridine (30ml) chloroform (100ml) mixture for two days at 25°C after the initial reaction was finished to give a light brown suspended solid which on separation gave a brown syrup containing 7.08% sulphur. T.L.C. (cellulose, solvent (C), developer (I) showed compounds with R_g = 0.73 and 1.44.

Glucuronic acid (4g) was treated with SO_2Cl_2 (12ml) in pyridine (30ml) chloroform (100ml) mixture for four days at 35°C after the initial reaction to yield a black brown oil. T.L.C. (cellulose, solvent (C), developer (I)) showed only a single spot R_g = 0.877.

Glucuronic acid (4g) was treated with SO_2Cl_2 (6.5ml) in pyridine (30ml) chloroform (100ml) mixture for four days at 50°C after the initial reaction to yield a black tar.

Glucuronic acid (4g) was treated with SO_2Cl_2 (7ml) in pyridine (30ml) chloroform (100ml) mixture for two days at 25°C after the initial reaction to yield a crystalline product containing 10.6% sulphur. Recrystallisation from acetone gave a product with 17.7% sulphur. T.L.C. (cellulose, solvent (C), developer (I)) showed the presence of a compound with R_g = 3.18.

Glucuronic acid (4g) was treated with SO_2Cl_2 (7.5ml) in pyridine (30ml) chloroform (100ml) mixture for three days at 25°C to give a crystalline product containing 17.75% sulphur after recrystallisation from acetone.

Glucuronic acid (4g) was treated with SO_2Cl_2 (7ml) in pyridine (30ml) chloroform (100ml) mixture for four days at 25°C to give a crystalline product which was recrystallised from acetone. Analysis gave C = 38.03%, H = 4.85%, S = 18.12% and N = 7.45%. Dissolving in methanol and evaporation to the start of precipitation of a crystalline material, filtering and washing with a little methanol gave a crystalline solid containing 18.41% sulphur.

Glucuronic acid (4g) was treated with SO_2Cl_2 (8ml) in pyridine (30ml) chloroform (100ml) mixture at -5°C until all the SO_2Cl_2 had been added then reacted at 25°C for two days. The reaction mixture was filtered and the filtrate poured in to ice cold 15% H_2SO_4 and stirred until the two phases were clear on standing. The chloroform layer was separated, washed with dilute H_2SO_4, water, saturated sodium bicarbonate solution, water and dried over Na_2SO_4 (anhydrous) then evaporated (45°C, 15mm) to a syrup. Analysis gave C = 32.57%, H = 3.34%, S = 4.16%, N = 2.39%, Cl = 28.69%. The aqueous layer was neutralised with $BaCO_3$, filtered, evaporated to 200ml in the presence of $BaCo_3$, filtered, passed down a column of ion exchange resin (zeoCarb 225), neutralised with $BaC\theta_3$, filtered and evaporated to dryness. The residue was extracted with 85% ethanol, evaporated to dryness, dissolved in hot 95% ethanol, filtered and evaporated to dryness. Analysis gave S = 9.33%, C = 66.1%, H = 2.9%, Cl= 3.39%, Ba = 4.02%. The residue from the first ethanol extraction was extracted with water, filtered and evaporated to dryness. Analysis gave C = 25.13%, H = 1.57%, Cl = 20.18%, Ba = 39.95%. The residue from this extraction was dried and analysed as C = 42.06%, H = 5%, S = 0.99%.

Galacturonic acid (4g) was treated with SO_2Cl_2 (8ml) in pyridine (30ml) chloroform (100ml) mixture at -5°C until all the SO_2Cl_2 had been added then reacted for four days at 25°C. The reaction mixture was filtered and the filtrate poured in to ice cold 15% H_2SO_4 and stirred until the layers cleared. The chloroform layer was separated, washed with dilute H_2SO_4, water, saturated $NaHCO_3$ solution, water and dried over Na_2SO_4

(anhydrous) then evaporated (45°C, 15mm) to a syrup (2ml). On standing at room temperature a crystalline solid formed which was recovered free from liquor by pressing between Whatman No.541 filter paper. The crystalline solid was washed with a few drops of ethanol after transfer to fresh paper, dried and analysed C = 31.76%, H = 4%, S = 16.44%, N = 5.92%. The liquor was recovered from the paper by extraction with ethanol and evaporated to a syrup. Analysis C = 30.99%, H = 3.88%, S = 15.51%, N = 5.92%. The aqueous layer was neutralised with $BaCO_3$, filtered, concentrated to 200ml, passed down an ion exchange column (ZeoCarb 225), neutralised with $BaCO_3$, filtered, evaporated to dryness, extracted with 85% ethanol, filtered and evaporated to dryness. Analysis C = 68.72%, H = 2.29%, S = 0%.

Hydrolysis of pectin

1N NaOH (200ml) was slowly added to a dispersion of pectin (100g) in water (1 L). When solution was complete the liquor was warmed to 40°C and pectinase (10g) added. The mixture, in a 2L flask, was covered by a layer of toluene and allowed to stand in a thermostatted water bath at 40°C for ten days. The solution was filtered and the filtrate passed down a column of ZeoCarb 225 ion exchange resin. The resin was washed with 200ml water and the washings combined with the main solution. The solution was treated with animal charcoal and filtered. The solution was neutralised with $CaCO_3$, filtered and evaporated to 500 ml, retreated with ZeoCarb 225 and the eluant evaporated

to dryness. The galacturonolactone was dried and stored over P205.

Sulphation of pectin

Dry Pectin (4g) suspended in pyridine (30ml) chloroform (100ml) mixture and cooled to 0°C. Sulphuryl chloride (7ml) was added dropwise to the stirred mixture at such a rate that the reaction temperature was maintained between 0°C and 10°C. After the addition was complete the reaction was continued for 2 days at ambient temperature. No apparent solution of the pectin had occurred and the pectin was removed by filtration and the pectin washed with 3 x 100ml portions of chloroform then dried under vacuum over P205. The product contained 0.8% sulphur.

Pectin (4g) in pyridine (30ml) chloroform (100ml) mixture was treated with SO_2Cl_2 (8ml) as in the previous example but for 4 days at ambient temperature. The product had a sulphur content of 1.1% and 0.8% of chlorine.

Pectin (4g) in pyridine (30ml) chloroform (100ml) mixture was reacted with SO_2Cl_2 (8ml) as in the previous example for 4 days at 40°C. The product had a sulphur content of 2.89% and 1.13% of chlorine. Infra red analysis showed adsorption bands at 1230 cm^{-1}, 890 cm^{-1} and 835 cm^{-1} (IRT 8).

Hyrdolysis of alginic acid

Alginic acid (100g) was wet with ethanol (50ml) in a 3L flask then 88% formic acid (2L) added and the mixture dispersed by shaking. The mixture was then stirred for 16 hours and refluxed with stirring for a further 24 hours. After cooling

the mixture was filtered and a large quantity of black-brown cake of unhydrolysed starting material obtained. This was returned to the liquor, dispersed by shaking, and the reflux continued for a further 24 hours. After cooling and filtering a large amount of unhydrolysed material remained and was discarded. The filtrate was concentrated under reduced pressure to a syrup. 95% ethanol (1L) was added to the syrup and the mixture heated with shaking at 65-70°C for 30 minutes. The mixture was then concentrated to a syrup under reduced pressure. The syrup was then refluxed with 95% ethanol (2L) in an attempt to dissolve it. On the addition of the ethanol a light brown solid formed which did not dissolve in ethanol on prolonged heating (after separation from the main solution). The ethanol solution was filtered, heated with animal charcoal, filtered and concentrated under reduced pressure to a syrup. Absolute ethanol (20ml) was stirred in to the syrup and the mixture allowed to stand at room temperature for 24 hours an d then at 5°C for a week. No crystalline material was obtained. The alcohol was removed under reduced pressure and a small quantity was then charged to a formate buffered anionic exchange column (Permutite SRA 101). Elution of the column with 0, 0.1, 0.2, 0.5, L and 2 N formic acid in steps of 50ml gave a series of fractions which when examined by paper chromatography using eluant (C) and developer (I) showed a considerable amount of 'streaking'. The chromatogram indicated that the 1N formic acid fraction probably contained the mannuronolactone and this fraction on evaporation gave a crystalline solid which was used

to 'seed' the main syrup. After standing at 5°C for three months the syrup developed a mass of crystals. Separation of a sample of the crystalline material from the residual liquoe was achieved by pressing between filter paper, removing the slightly brown coloured crystals to fresh paper and washing with a small amount of acetone-ethanol mixture (70:30 v/v).

Sulphation of alginic acid

Dry alginic acid (4g) suspended in pyridine (30ml) chloroform (100ml) mixture was treated with SO_2Cl_2 (8ml) as described for pectin for 2 days at ambient temperature. The reaction product was insoluble in the reaction medium and the alginic acid product was recovered by filtration, washed with 3 x 100ml portions of chloroform and dried in vacuo over P205. The product had a sulphur content of 0.8% and 2.97% of chlorine.

Alginic acid (4) suspended in pyridine (30ml) chloroform (100ml) mixture was reacted with SO_2Cl_2 (8ml) as previously described for 4 days at ambient temperature. The chloroform washed and dried product had a sulphur content of 7.99% Infra red analysis showed some evidence of the sulphate group being present by adsorption bands at 1230 cm^{-1}, 870 cm^{-1} and 810 cm^{-1} (IRT 9).

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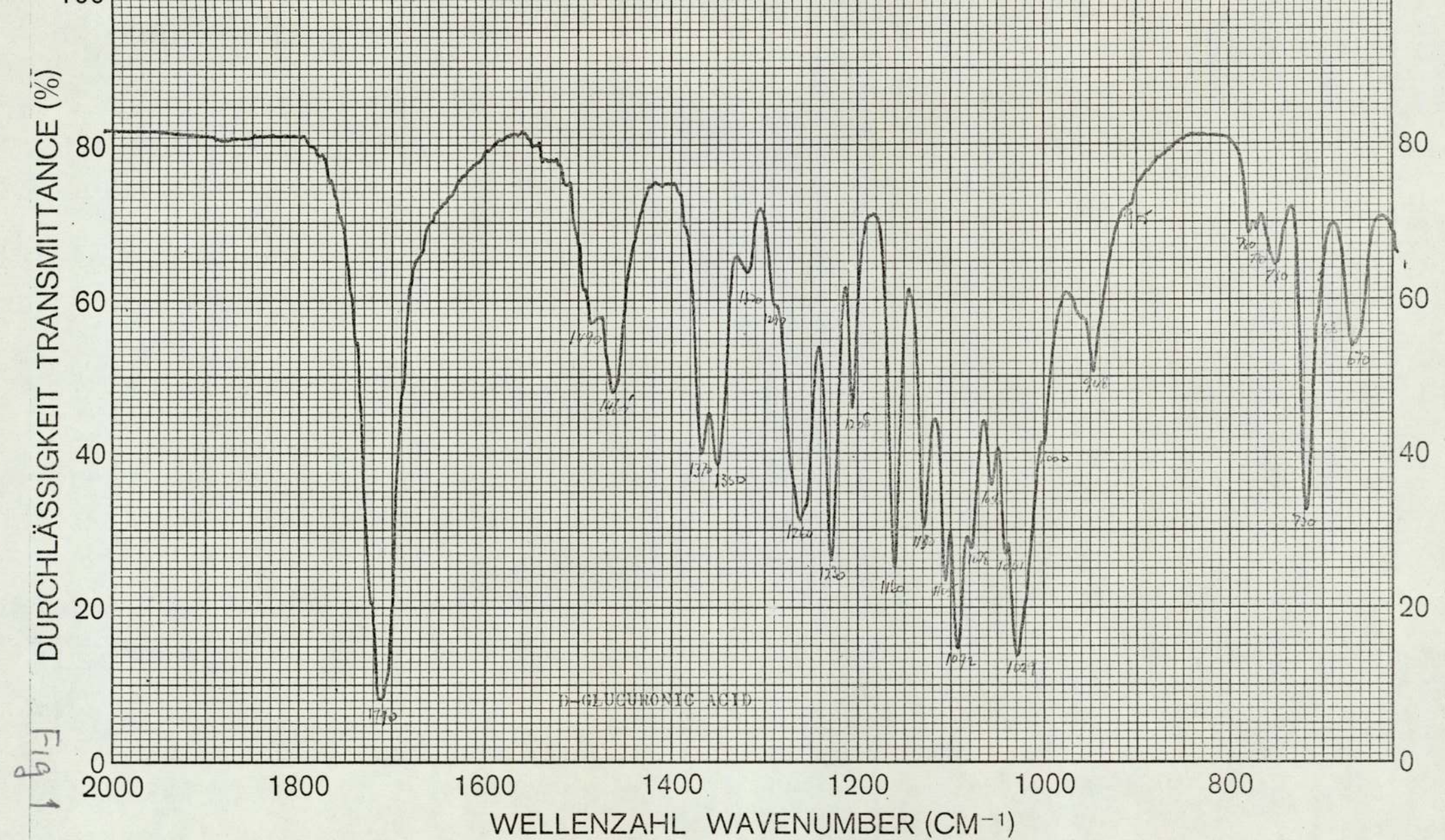
Legend

Infra red spectra ($2000cm^{-1}$ - $600cm^{-1}$)

Figure 1	Glucuronic acid	
2	Galacturonic acid	
3	Galacturonic acid sulphate	IRT 1
4	" " "	IRT 2
5	Glucuronic acid sulphate	IRT 3
6	" " "	IRT 4
7	Pectin sulphate	IRT 8
8	Alginic acid sulphate	IRT 9

NMR Spectra

Figure 9	Glucuronic acid	
10	Galacturonic acid	
11	" "	4τ to 7τ (100MHz)
12	Mannuronic acid	
13	" "	4τ to 6τ (100MHz)



PROBE SAMPLE D-Glucuronic Acid. (Koch-light)	KURVE NR. CURVE NO. -	GESCHWINDIGKEIT SCAN SPEED 8	BEARBEITER OPERATOR Y.
HERKUNFT ORIGIN Dr. Holt.	KONZENTRATION CONC approx. 1.0%	SPALT SLIT Standard	DATUM DATE 28.5.69
LÖSUNGSMITTEL	SCHICHTDICKE CELL PATH 13.0 mm/m. dia. disc.	BEMERKUNGEN REMARKS	
	VERGLEICH		

NO.

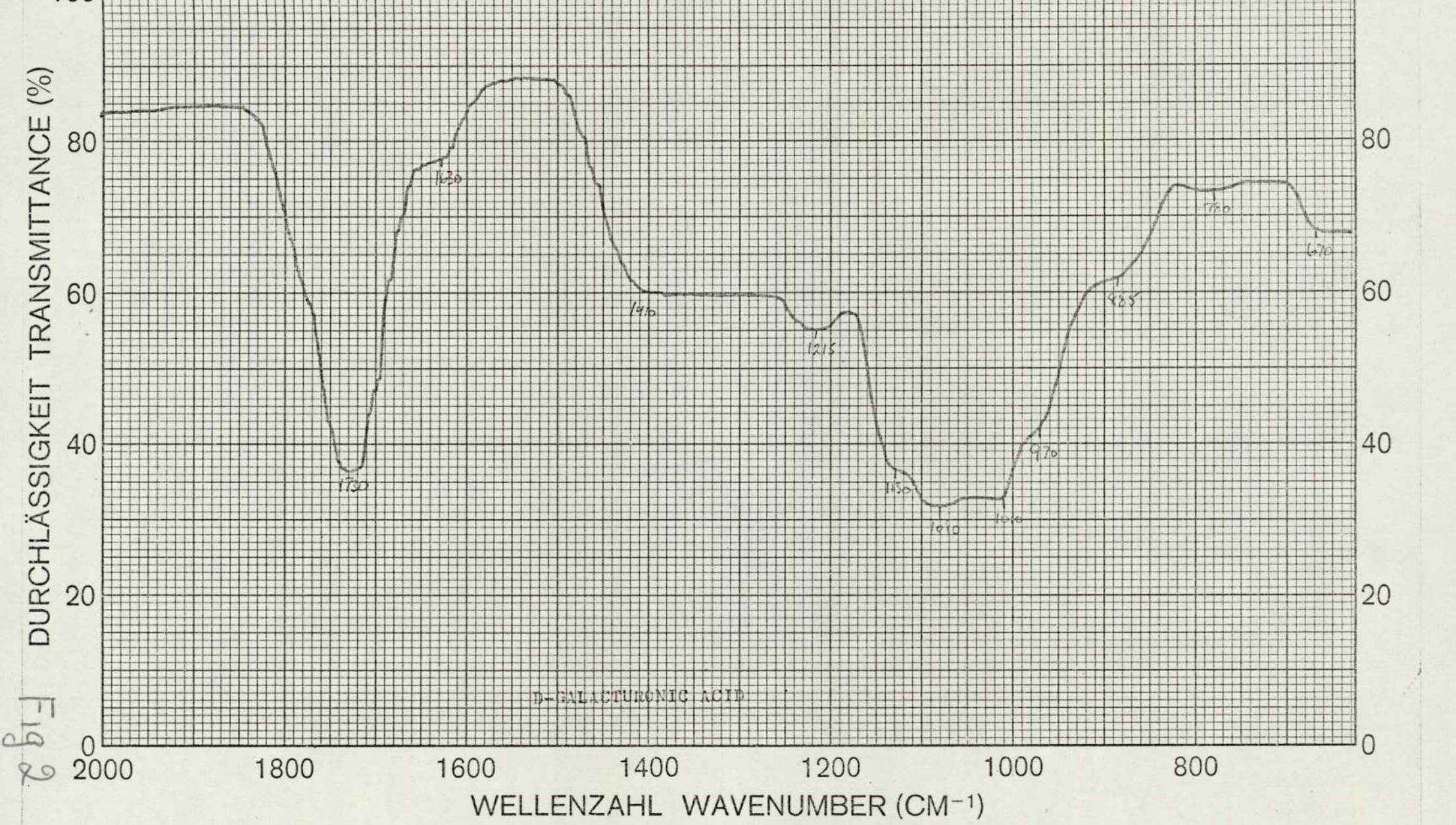
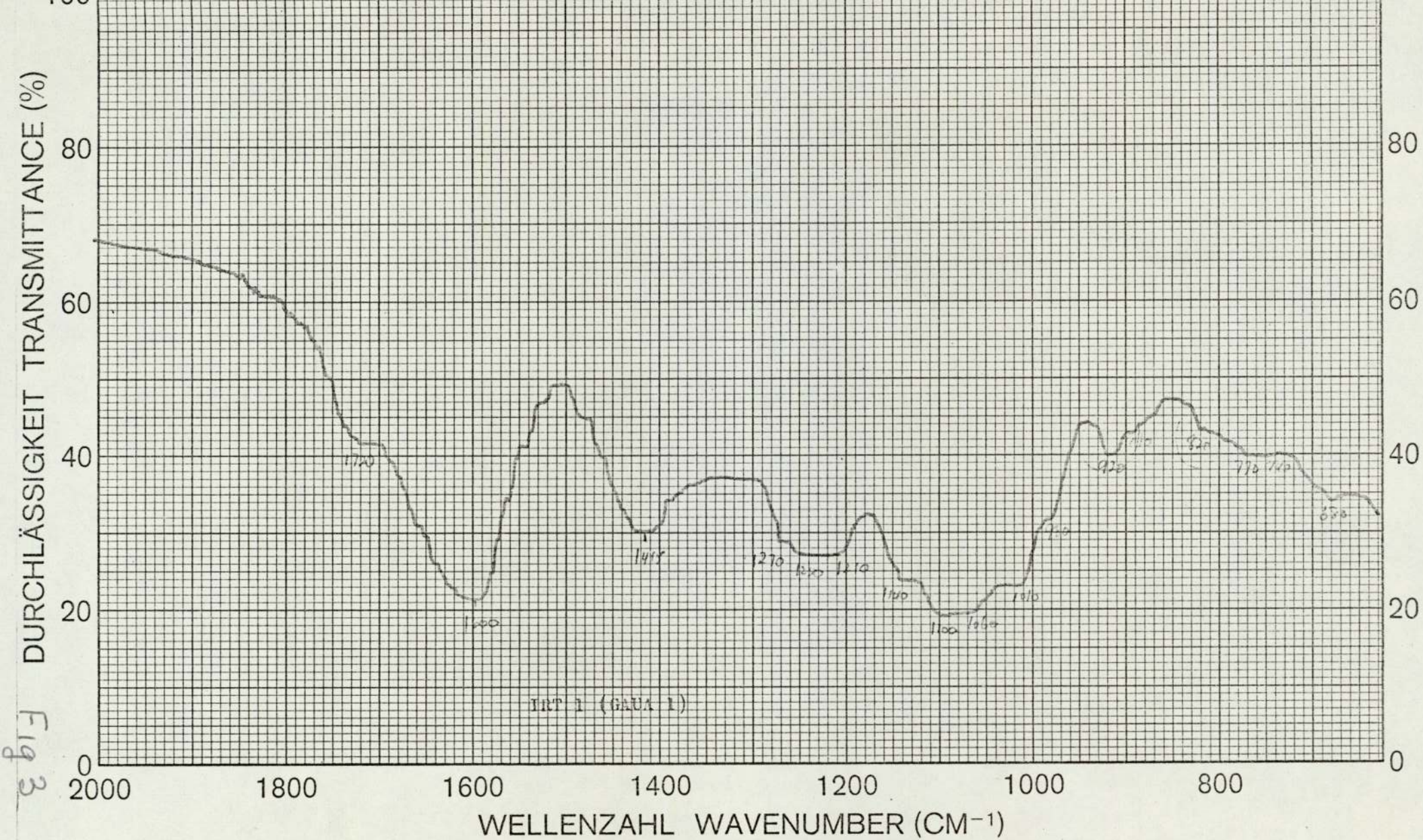


Fig 2

D-GALACTURONIC ACID

PROBE SAMPLE Galacturonic acid	KURVE NR. CURVE NO.	GESCHWINDIGKEIT SCAN SPEED	BEARBEITER OPERATOR
	KONZENTRATION CONC	SPALT SLIT	DATUM DATE
HERKUNFT ORIGIN	SCHICHTDICKE CELL PATH	BEMERKUNGEN REMARKS	
LÖSUNGSMITTEL	VERGLEICH		

NO.



PROBE SAMPLE GAUA 1.	KURVE NR. CURVE NO. -	GESCHWINDIGKEIT SCAN SPEED	BEARBEITER OPERATOR
	KONZENTRATION CONC approx. 1.0 %.	SPALT SLIT Standard.	DATUM DATE 10.9.70
HERKUNFT ORIGIN A. Stokes.	SCHICHTDICKE CELL PATH 13.0 %. dia. disc.	BEMERKUNGEN REMARKS	
LÖSUNGSMITTEL	VERGLEICH		

NO.

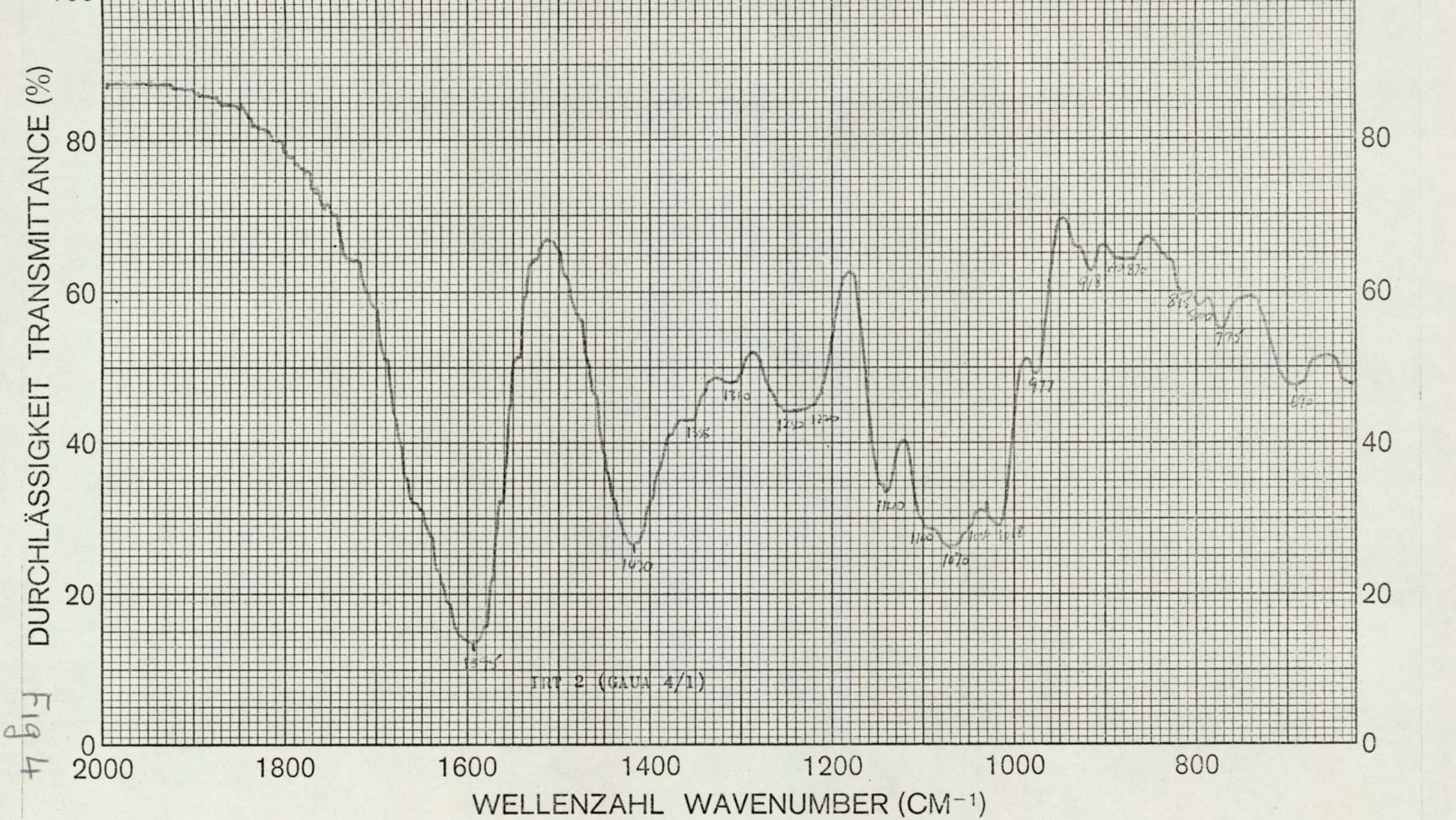


Fig 4

PROBE SAMPLE Gaua 4/1.	KURVE NR. CURVE NO. -	GESCHWINDIGKEIT SCAN SPEED x	BEARBEITER OPERATOR H
	KONZENTRATION CONC. appx. 1.0%	SPALT SLIT Standard.	DATUM DATE 1.9.72
HERKUNFT ORIGIN A. Stokes.	SCHICHTDICKE CELL PATH 13.2 mm. dia. disc.	BEMERKUNGEN REMARKS	
LÖSUNGSMITTEL	VERGLEICH		

NO.

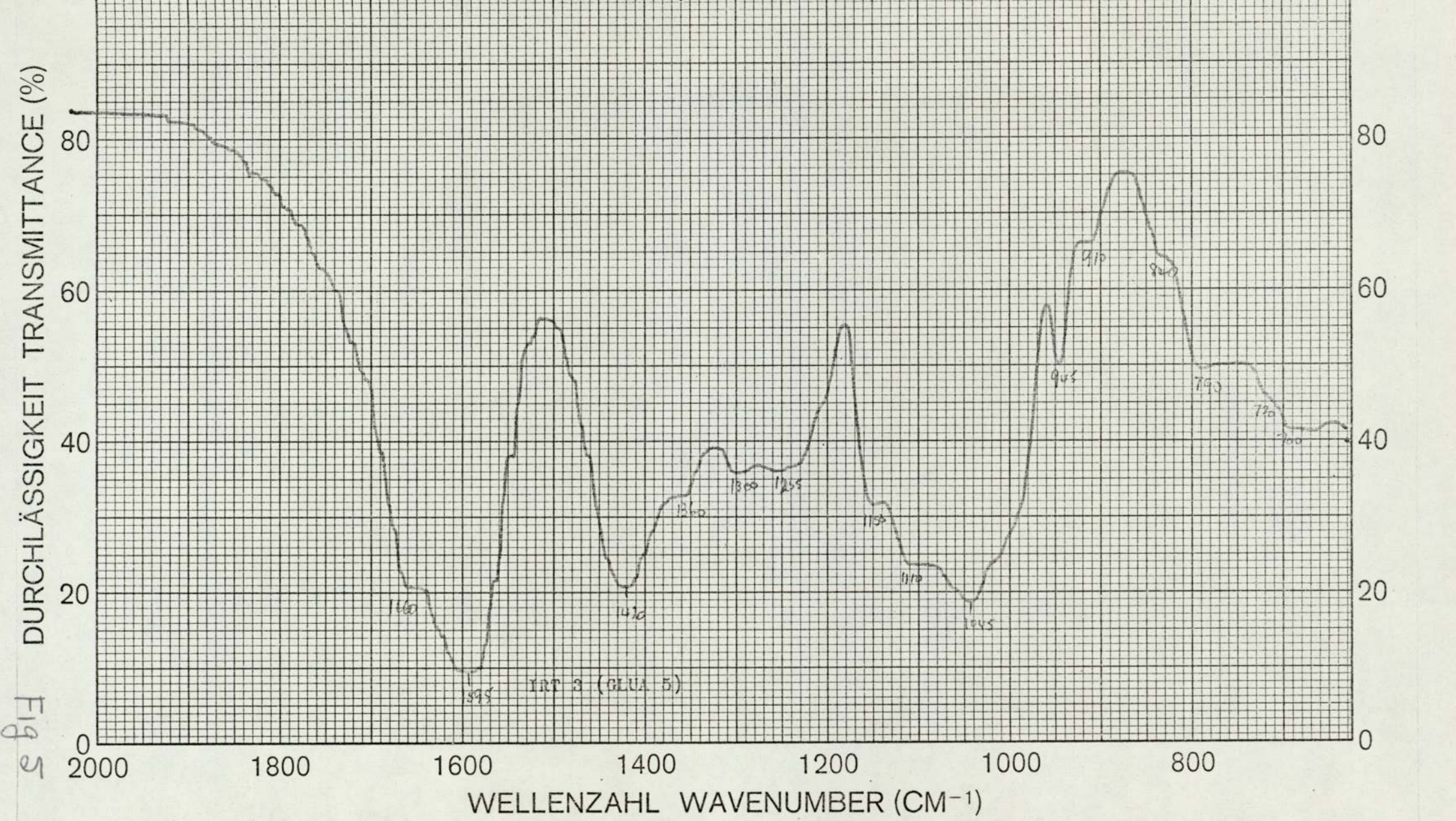
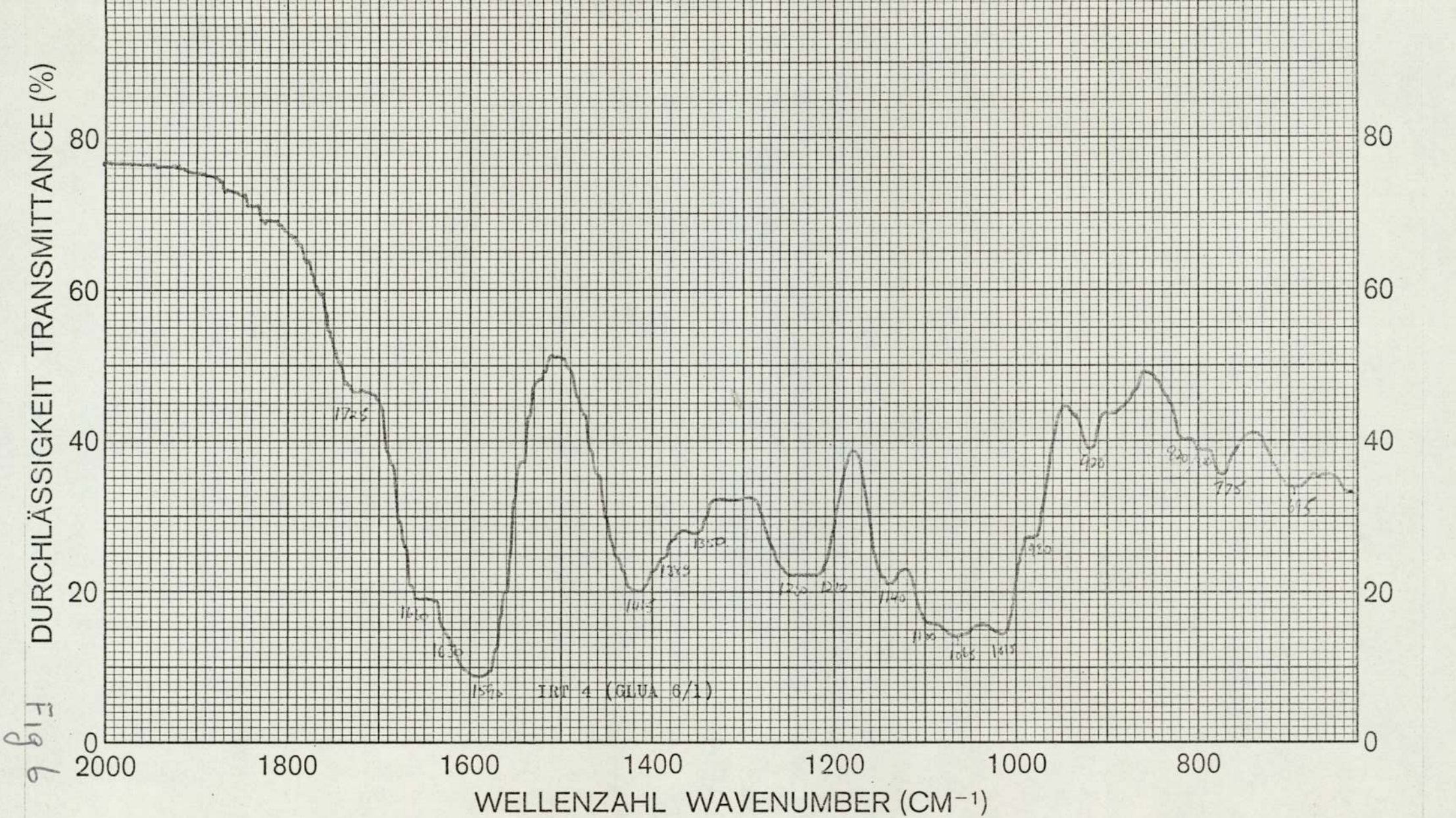


Fig 5

PROBE SAMPLE Glua 5.	KURVE NR. CURVE NO.	GESCHWINDIGKEIT SCAN SPEED	BEARBEITER OPERATOR
	KONZENTRATION CONC approx. 1.0%	SPALT SLIT	DATUM DATE
HERKUNFT ORIGIN A. Stokes.	SCHICHTDICKE CELL PATH 12 mm. dia. disc.	BEMERKUNGEN REMARKS	
LÖSUNGSMITTEL	VERGLEICH		

NO.



9 817

PROBE SAMPLE GLUA 6/1.	KURVE NR. CURVE NO.	GESCHWINDIGKEIT SCAN SPEED	BEARBEITER OPERATOR
	KONZENTRATION CONC. approx. 1:5 2.	SPALT SLIT	DATUM DATE
HERKUNFT ORIGIN A. Stokes.	SCHICHTDICKE CELL PATH 13.3 mm/n. dia. disc.	BEMERKUNGEN REMARKS	
LÖSUNGSMITTEL KB	VERGLEICH		

NO.

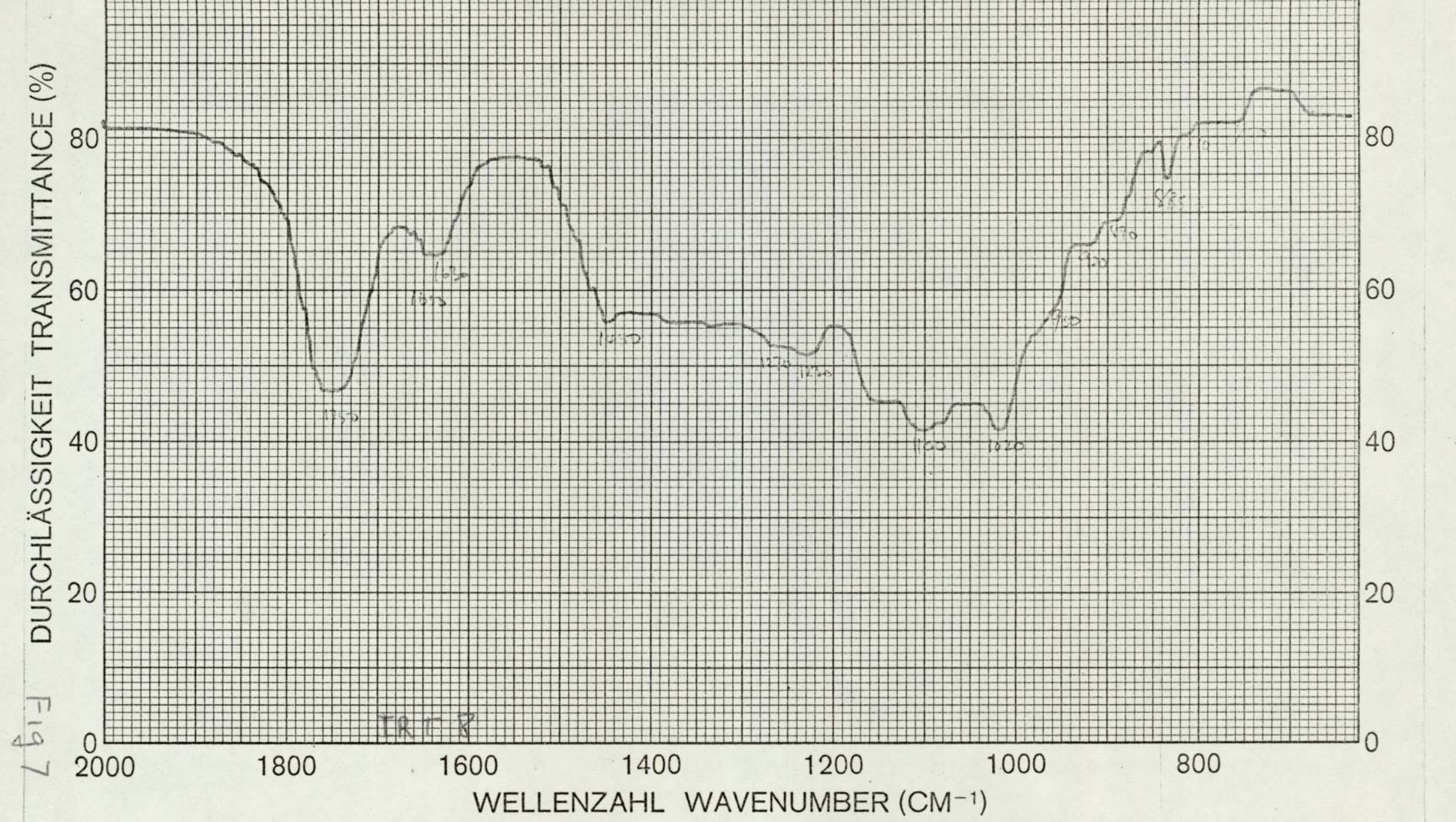


Fig 7

PROBE SAMPLE Pect 37	KURVE NR. CURVE NO. -	GESCHWINDIGKEIT SCAN SPEED 8	BEARBEITER OPERATOR VC
	KONZENTRATION CONC. approx. 2-3%	SPALT SLIT Standard	DATUM DATE 2.4.71.
HERKUNFT ORIGIN A. Stokes / D. Holt.	SCHICHTDICKE CELL PATH 13.0 m/m. dia. dix	BEMERKUNGEN REMARKS	
LÖSUNGSMITTEL SOLVENT KB	VERGLEICH REFERENCE Air		

NO. EE 6

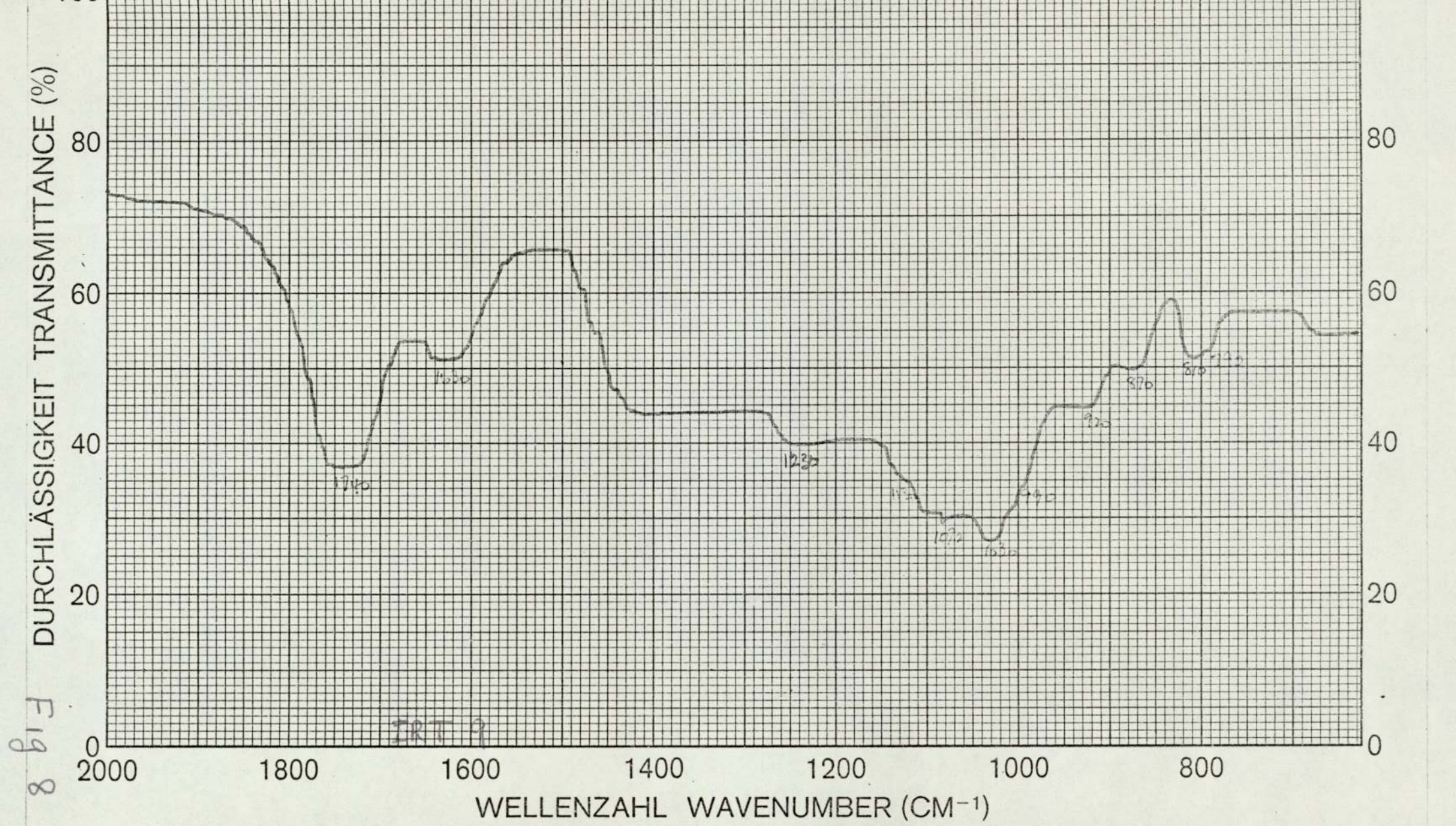


Fig 8

PROBE SAMPLE: Alg 34.	KURVE NR. CURVE NO.: -	GESCHWINDIGKEIT SCAN SPEED: 8	BEARBEITER OPERATOR: VC.
HERKUNFT ORIGIN: A. Stokes / Dr. Holt.	KONZENTRATION CONC.: approx. 2·0%	SPALT SLIT: Standard.	DATUM DATE: 1·4·71
LÖSUNGSMITTEL: KB.	SCHICHTDICKE CELL PATH: 13·0 cm/m. dia disc	BEMERKUNGEN REMARKS:	
	VERGLEICH: Air		

NO. 62.

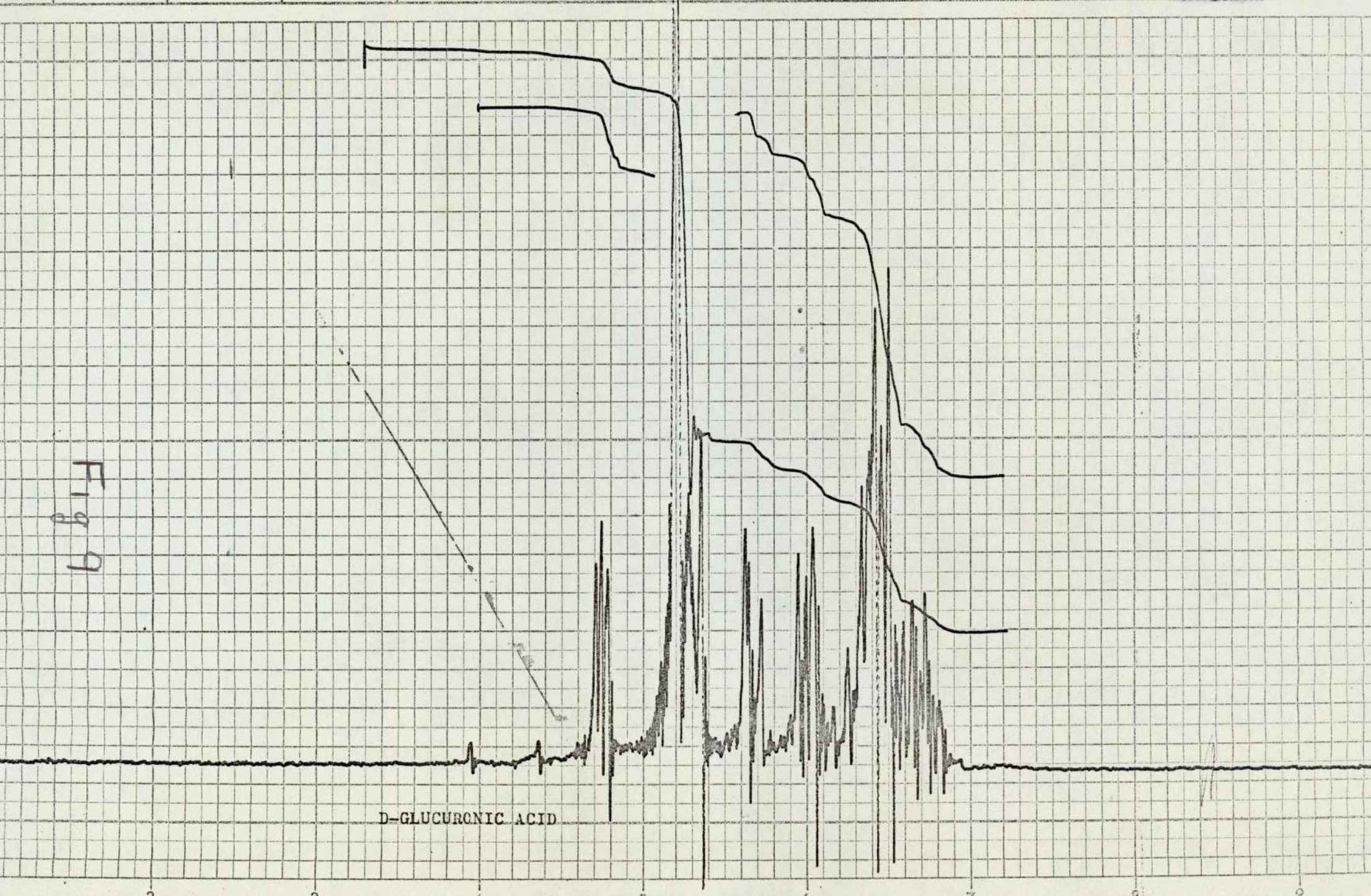


Fig 9
D-GLUCURONIC ACID

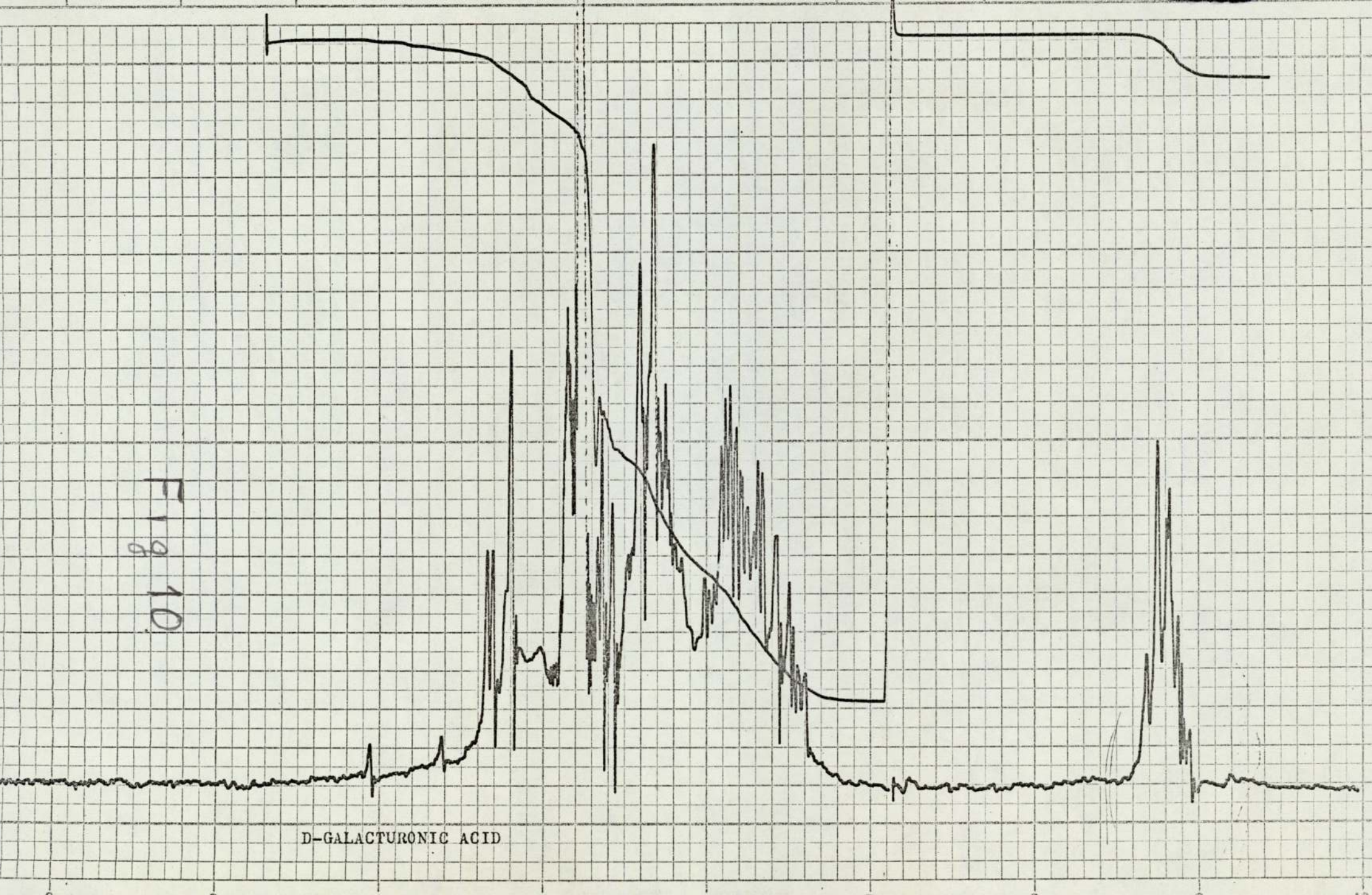
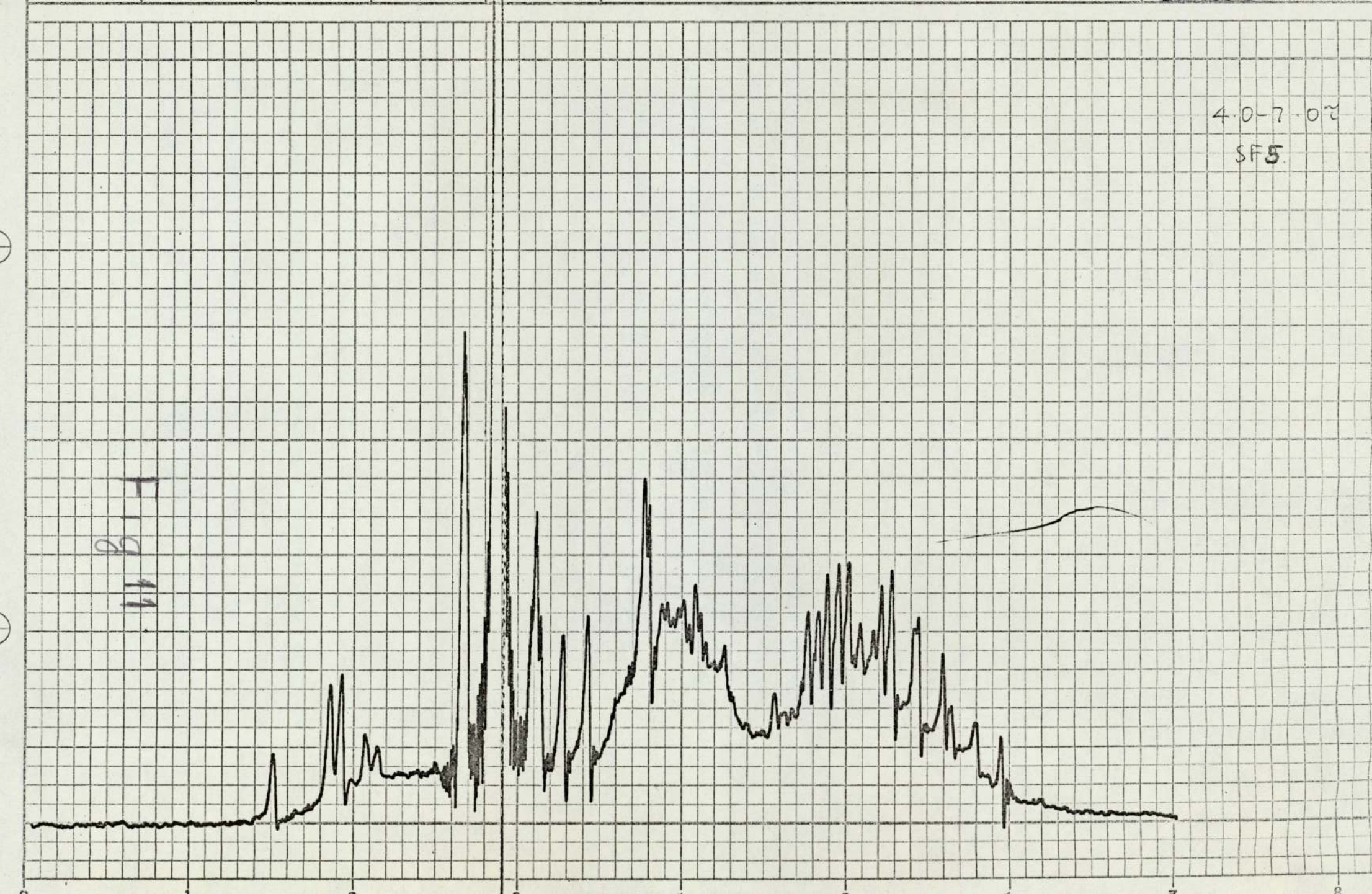


Fig 10

D-GALACTURONIC ACID


4-0-7-07
SF5
F1 22 44

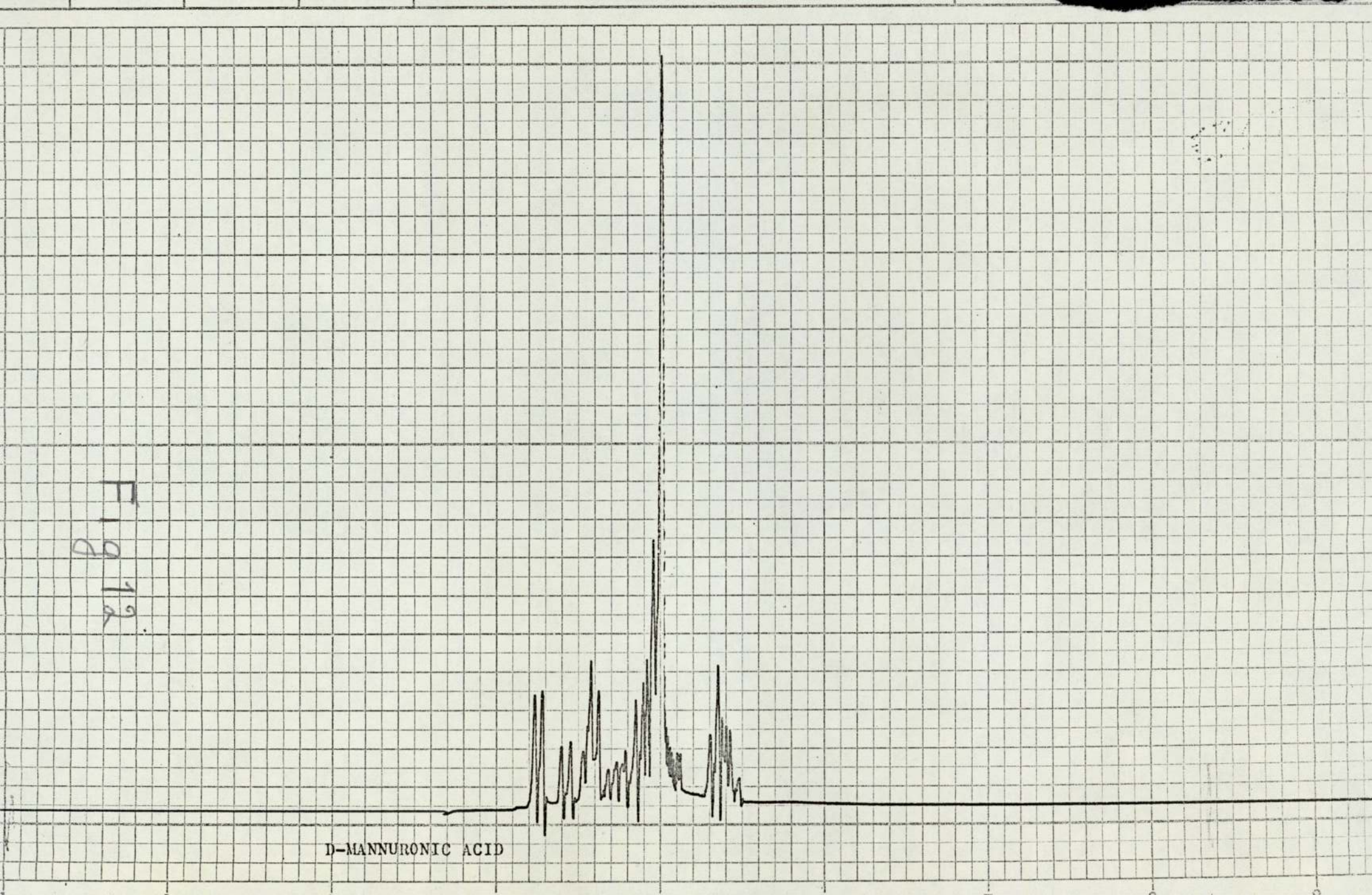


FIG 12

D-MANNURONIC ACID

SF2

Sens. 1.

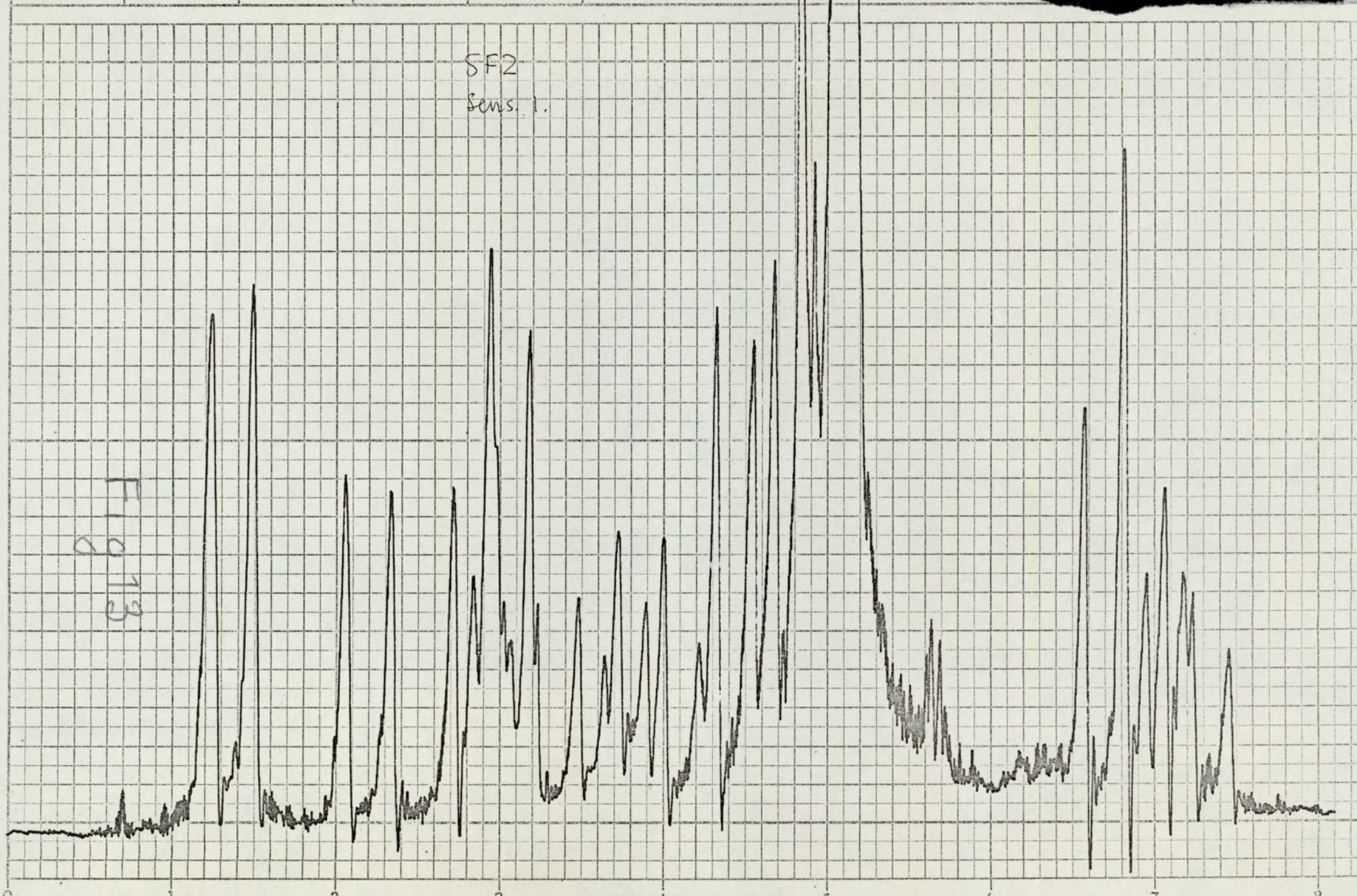


Fig 13