A NEW MICRO-ANALYTICAL SYSTEM FOR REDUCING SUGARS - APPLICATIONS TO SEDIMENT AND SEAWATER

by

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ABSTRACT

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Submitted to the Department of Earth and Planetary Sciences on September 1, 1970 in partial fulfillment of the requirement for the degree of Master of Science.

The distribution of carbohydrates in the marine environment has been largely ignored by investigators in the field of organic geochemistry. The primary hindrance to this type of research has been the lack of appropriate analytical techniques to 1) quantitatively extract sugars from natural samples and 2) separate and identify these sugars.

The latter difficulty has been resolved by construction of an automatic sugar analyzer which is: 1) rapid-monosaccharide mixtures are fully resolved in three to four hours; 2) highly sensitive- 10^{-10} to 10^{-11} moles of sugar are detected; 3) capable of excellent reproducibility; and 4) capable of yielding high resolution.

The design of the sugar analyzer system is based on an automatic amino acid analyzer, however totally different columns and stains are employed. During the construction of this new system many parameters had to be adjusted, including: 1) resin-type and particle size; 2) length and diameter of resin bed; 3) temperature of the column; 4) type of eluant; 5) pumping mechanisms; 6) pumping rates; and 7) stains.

The completed system separates sugars by pumping 89% ethanol under pressure (400 psi) through a long narrow column (110 x .28 cm) packed with an extremely fine-grained, strongly anionic resin in the sulfate form (Technicon Type S Resin, 20 microns). The eluted sugars are stained during reaction with an alkaline solution of tetrazolium 'blue'. This dye is considerably more sensitive and less corrosive than dyes used by other investigators. Until now it had not been employed in analytical systems for sugars because of the concomitant precipitation of diformazan which clogs capillary tubing. However, extensive laboratory experimentation by this author have succeeded in rendering tetrazolium 'blue' applicable.

This sugar analyzer has a three-sample-a-day capacity, however the output will be tripled by construction of additional columns. Furthermore, all peaks recorded by the spectrophotometer will soon be automatically integrated and digitized in order to facilitate the quantification of large amounts of data.

Prior to analyzing carbohydrates in seawater and sediment samples, extraction procedures were tested with standard sugar solutions in order to determine if: 1) all sugars were quantitatively extracted; and 2) synthesis or destruction of sugars were occurring. Adequate procedures were finally

established for the extraction of free sugars from seawater, sediments and seston; and of combined sugars from seawater and seston.

Of the several problems that remain one that is particularly relevant is presently being investigated in our laboratory. Seawater samples are usually de-salted on ion-exchange resins. However, in this study it became obvious that ion-exchange techniques led to almost complete destruction of one sugar, desoxyribose, and partial destruction of a second, ribose. Infra-red spectroscopy has been, and will continue to be, employed to study in detail the perseverance and/or alterations in sugars during ion-exchange desalting.

Extraction of combined sugars from sediments proved to be exceedingly difficult. Apparently mineral surfaces in the sediment catalyze the destruction and molecular re-arrangement of monosaccharides released during acid hydrolysis of polysaccharides. Enzymatic hydrolysis techniques are being examined as a viable alternative.

Additional evidence for mineral surface-sugar interactions was observed after a slurry of ignited sediment (800°C for three hours) and standard sugar solutions were refluxed for 16 hours. Lyxose and one as yet unidentified sugar were produced by molecular re-arrangement of the pre-existing sugars. Further research on this process is being pursued in our laboratory. In addition, synthesis of sugars from simple organic molecules (e.g. glycerine and formaldehyde) on clays is being attempted.

The first quantitative analyses for free monosaccharides in Black Sea sediments indicates that these sugars represent several percent of the total organic matter. Furthermore, comparison of these data with analyses of free sugars in lake sediment (Vallentyne and Whittaker,1957) suggests that depositional environment is definitely linked with the sugar content of the sediment. Therefore, sediments from different environments (open ocean, polluted and unpolluted estuaries, rivers, and salt marshes) are presently being analyzed to test this idea.

Preliminary laboratory experiments indicate that the free sugar concentration of lake and seawater affects the growth of some plankton species. Plankton density in a water body may likewise affect the concentrations of free sugars there. Therefore, detailed studies of the sugar content of dissolved and particulate organic matter from several areas of high biologic productivity are being initiated in order to examine such interrelationships.

It is known that carbohydrates account for 60 to 70% of the organic matter in domestic and industrial wastes. The new sugar analyzer system is an ideal apparatus for measuring and identifying the sugar content of polluted areas, such as the effluent of the Revere Sugar Company, Boston, Massachusetts and the sewage disposal area in New York Bight.

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PART ONE

I ANALYTICAL SYSTEM-AUTOMATIC ANALYSIS OF MICROGRAM QUANTITIES OF MONOSACCHARIDES BY COLUMN CHROMATOGRAPHY

INTRODUCTION

In September, 1969, at the Woods Hole Oceanographic Institution, a study of the distribution of free and combined carbohydrates in the marine environment was initiated. For this study it was necessary to construct an analytical system to: 1) separate sugar mixtures into component monosaccharides; and 2) identify these sugars.

Since monosaccharides demonstrate similar chemical and physical properties, the usual fractionation techniques (e.g., fractional crystallization) are inadequate for separating complex sugar mixtures. Therefore several chromatographic techniques, including paper, thin-layer, gas-liquid, and column chromatography were examined. Of these, column chromatography was found most desirable, since it could be readily adapted to the automatic separation and quantification of sugars.

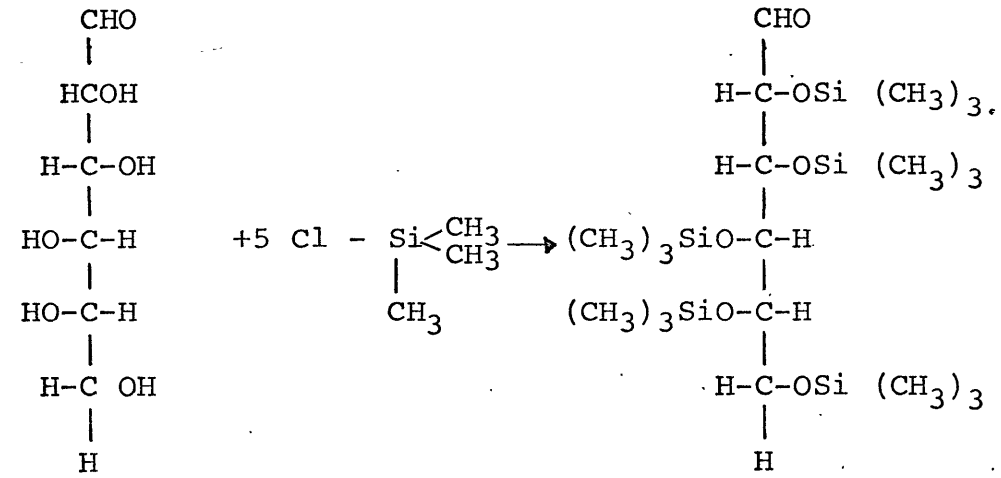
Construction of the automatic sugar analyzer is described in the following section.

II ANALYTICAL TECHNIQUES CONSIDERED PRIOR TO THE CONSTRUCTION OF THE AUTOMATIC SUGAR ANALYZER - BACKGROUND

1. Gas Liquid Chromatography:

A gas chromatograph is designed to separate mixtures of readily volatile compounds. Although sugars are not volatile, by forming volatile derivatives these compounds can be analyzed. Derivatives are formed by reacting appropriate compounds with the hydroxyl groups on the sugar molecule (Hammarstrand and Bonelli, 1968). Some derivatives are methyl ethers, actates, acetals (or ketals) and trimethylsilyls (Wells, et al. 1964). The trimethylsilyl (abbreviated TMS) derivatives are commonly used. The process of forming TMS derivatives is called silylation and is illustrated below.

(1)



Silylation is rapid (about five minutes at 25°C) and also quantitative (Sweeley, et al. 1963). The silylated solution is injected directly into the gas chromatograph. The separation of five sugars is illustrated in Figure 1.

Although this method appears simple and also highly sensitive (nanogram quantities are detected), many complications arise. For example, the main advantage of gas chromatography (i.e., its extreme sensitivity) is also the main disadvantage. Volatile contaminants will show up on the chromatogram and possibly interfere with sugar peaks. For instance, Blumer (1965) found organic compounds coming from laboratory air ventilator filters to be significant sources of contamination in his G. C. analyses. In addition, natural samples (i.e., sediment, sea water, etc.) contain volatile compounds (hydrocarbons, aromatics) which may also interfere. However, even when a standard sugar solution is silylated, a complex chromatogram results. Figure 1 illustrates the separation of five sugars; fourteen peaks are present. The complication arises from the fact that alpha and beta anomers of sugar enantiomorphs are detected. This is especially significant for galactose (see Figure 1). Since both L and D enantiomorphs occur naturally, four peaks will result: α-L-

galactose, β -L-galactose, ∝ -D-galactose and β -D-galactose. In addition, even when only one anomer of a sugar is present, most silylation techniques cause anomerization to occur to varying degrees (Wells, et al. 1963). However, a technique involving the use of hexamethyldisilazane for silylation has been recently developed which minimizes anomerization (David Boylan, pers. comm., 1969).

Derivatives of some sugar enantiomorphs are difficult to separate; peaks 3 and 4, 9 and 10, and 12 and 13 in Figure 1 are not resolved. This makes the problem of peak identification difficult since the use of the usual chromatographic parameter, the retention time, is not reliable enough to identify peaks in a complex chromatogram (especially when contaminants are present). Therefore, an alternate method for peak identification must be employed: mass-spectrometry. However, mass-spectrums of TMS derivatives are complex because a large number of fragmentations occur (Figure 2). Interpretations of these spectra are difficult and time consuming.

For the reasons given above, I decided that the technique of gas chromatography is not suitable for the separation and identification of unknown mixtures of sugars.

2. Paper Chromatography:

Paper chromatography is an old, yet reliable method for separating complex mixtures into their components. Sugars are separated in a one-dimensional descending run which has the advantage that many samples plus standards can be spotted on the same sheet and can be analyzed simultaneously.

Paper chromatography has several short-comings. For instance, bulky developing tanks are required; pyridine, which is commonly used in the mobile phase (Degens and Reuter, 1964), is an unpleasant reagent to handle (its vapors are flammable, noxious and pungent); and long developing times are necessary (anywhere from 2 to 7 days depending upon the composition of the migrating solvent and the degree of separation required). Most dyes used to visualize the separated sugars will also stain the paper itself, thereby adding a background 'noise' which will vary in intensity depending upon the reactivity of the dye and the dyeing time and temperature selected. This background limits the sensitivity of the technique to the 1 to 5 microgram range (Whittaker and Vallentyne, 1957). In addition, quantification is difficult; crude estimates are made by running standards along side of samples and visually estimating spot size and density

(other methods will be described later). For these reasons, paper chromatography is not employed in the present analytical scheme.

3. Thin-Layer Chromatography:

The deficiencies of paper chromatography are resolved by thin-layer chromatographic techniques. The theory behind thin-layer chromatography (abbreviated TLC) is the same as that behind paper chromatography (as well as other chromatographic techniques). A mobile phase, a stationary phase, and a sample are present. Separation occurs because component compounds in the sample are preferentially retarded by the stationary phase by processes such as adsorption, partition, ion-exchange and molecular sieving (Brenner, et al. 1965).

TLC differs from paper chromatography in that the absorbent media can be varied. Typical absorbents are: silica gel, alumina, cellulose, and polyamide. These absorbents can be manually applied to a glass plate, or, pre-coated plastic sheets can be purchased.

The choice of the stationary phase and the mobile solvents is dependent upon the chemistry of the substances to be separated. Optimum separation conditions are established for each type of compound to be investigated. For example, partition chromatography is the most effective

mechanism for separating neutral and ionic hydrophilic compounds; i.e., carbohydrates, amino acids, peptides, sterols, vitamins, etc. A highly polar mobile phase is employed to dissolve and separate these compounds. Commonly water, methanol, ethyl acetate, and acetone are used in various proportions in the solvent (Kirchner, 1967).

Stains used to locate sugars on a paper chromatogram are employed in T.L.C. Degens and Reuter (1964) recommend the use four percent triphenyltetrazolium chloride (tetrazolium 'red') in methanol. Other reagents such as anilino-phosphoric acid (Medgysi, 1965), benzidine (Linskene, 1959) and anisaldehyde-sulfuric acid (Waldi, 1965) have been successfully employed to locate sugars on chromatograms. Some dyes are specific for ketoses, hexoses, uronic acids, sugar alcohols, and desoxysugars (Waldi, 1965). Commercially pre-coated TLC sheets containing fluorescent indicators are available. Sugars, as well as contaminants, show up as dark spots on a colored background under ultraviolet light. This method is not as sensitive and as selective as staining, however, the time saved makes its use desirable.

Silica gel does not react with the stains, therefore, when it is used as the stationary phase, background coloration is non-existent. This adds considerably to the

sensitivity (0.01 to 0.1 micrograms can be detected).

In addition to being more sensitive than paper chromatography, TLC separations are faster. With the proper choice of mobile and stationary phases, separations can be achieved within an hour after spotting. However, like paper chromatography, TLC is difficult to quantify. As before, crude estimates can be made by visually comparing spot size and density of the sample against a series of standards (20 to 30 percent accuracy, Whittaker and Vallentyne, 1957). When greater precision (in both TLC and paper chromatography) is required, several instrumental methods are available. Two examples are: photo-densitometric determination and elution-spectrophotometry. In the first method, the chromatograms are photographed by standardized photographic procedures and the negatives are then evaluated in a specially constructed optical integrator (densitometer). A calibration curve is constructed by plotting sample quantity versus spot density. This technique is most effective at low sample concentrations (< 0.2 ug/ml) where the calibration curves approach linearity. Under optimum conditions, accuracies of 5 to 10 percent can be achieved (Caster & Andrews, 1969).

In the second method (elution-spectrophotometry), the stained spots are cut out and eluted with a suitable solvent (i.e., water or aqueous ethanol) and the absorbances are measured on a spectrophotometer. Calibration curves of sample quantity versus absorbance are plotted. This technique is most effective at high concentrations ($>$ 1 μg/ml) because the color intensity of the extracted spots is lowered by the dilution necessary to fill the spectrophotometer cuvette. The accuracy is 5 to 10 precent (Gänshirt, 1965; and Dubois et al., 1956)

Both TLC and paper chromatography depend upon R_f values for qualitative determinations. R_f measurements are empirically determined as follows:-

$$R_f = \frac{\text{distance of center of spot from origin}}{\text{distance of solvent front from origin}}$$

Factors affecting the R_f values are: temperature, running distance, degree of chamber saturation, quality and nature of solvents used, quantity of sample applied, degree of activation of absorbent, thickness of absorbent layer, and technique of development (either ascending or descending). Therefore, strictly standardized conditions are required for reproducible R_f values (Bobbitt, 1963). Even then, positive identification is not assured unless standards are run on the chromatogram under identical conditions.

Presently, TLC is being employed as a qualitative check on column chromatography (see proceeding section). The use of two different chromatographic methods (with different stationary phases) assures the positive identification of the sugars present in the samples.

An analytical procedure for the TLC separation of sugars described by the Distillation Products Industries has been adapted. Eastman Chromatogram sheets 6061 (silica gel) 20 cm. long, are pretreated with a solution of sodium acetate in ethyl alcohol. (The sodium acetate and silica gel behave as the stationary phase.) After air drying, the sheet is: 1) activated at 100°C for 20 minutes; 2) spotted 2 centimeters from the lower edge with samples and standards; and 3) developed to a distance of 10 centimeters with acetic acid/chloroform/methanol/water (80:10:10:5). Visualization is achieved with an alkaline solution of p-anisyl tetrazolium chloride (or tetrazolium 'blue'). This dye is extremely sensitive; quantities on the order of 10^{-10} to 10^{-11} moles can be detected. (The reagent will be described in detail in a later section of the thesis.) A typical TLC separation is illustrated in Figure 3. The separation of several sugars in this chromatogram is poor. The resolution is improved, however, by a second development.

III AUTOMATIC SUGAR ANALYZER

The previous section indicated that analytical techniques for the separation of complex sugar mixtures (with subsequent detection) based on gas-liquid chromatography, paper chromatography, and thin-layer chromatography were undesirable for one, or several, of the following reasons: 1) insensitivity; 2) poor reproducibility; 3) insufficient resolution; 4) long analyses time; and 5) interferences by contaminants.

These problems were overcome by the author through the construction of a fully automatic analyzer capable of detecting 10^{-10} - 10^{-11} moles of sugar. A sample is loaded at the top of the column (filled with ion-exchange resin) and the sugars are separated, stained, and spectrophotometrically recorded within 3-4 hours.

The schematic of the auto-analyzer built by the author is depicted in Figure 4. Its design is based on an automatic amino acid analyzer (Moore and Stein, 1951; and Degens and Spencer, 1966).

Previously designed sugar analyzers (Jonsson and Samuelson, 1966; and Kesler, 1967) suffered from three flaws: 1) use of concentrated sulfuric acid as a major reagent; 2) low sensitivity; and 3) non-reproducible results.

Concentrated sulfuric acid is dangerous to manipulate and difficult to employ in an analytical system because of its corrosive effects. Jonsson and Samuelson (1966), for example, had to construct a teflon-covered piston for the reagent pump because the original steel piston became corroded while feeding the dye-sulfuric acid solution into the analyzing system.

The low sensitivity (the limit of detectability is 10^{-7} - 10^{-8} moles) is attributable to the dye mechanism. This is discussed in detail in the 'Dye' section.

Poor reproducibility was caused by the use of a multichannel peristalic pump (designed by the Technicon Corporation). The pump tubing rapidly became exhausted and was replaced at regular intervals resulting in variations in the flow rates which, in turn, affected the quantification of the chromatograms (Jonsson and Samuelson, 1966).

During the construction of the sugar analyzer, I have over avoided these problems through the careful manipulation of the following variables: 1) resin (type and particle size); 2) length and diameter of resin bed; 3) temperature of column; 4) type of eluant; 5) pumps (mechanical versus nitrogen pressure); 6) pumping rates; and 7) stains.

The difficulties encountered during the construction of the sugar analyzer are described in the following section, preceded by descriptions of the technical specifications of the instrument and procedures.

1. Technical Specifications and Procedures

a. Hardware

Figure 4 is a schematic diagram of the system used to analyze complex sugar mixtures. A nylon column having an inner diameter of 0.28 cm. was filled to a height of 110 cm. with Technicon Type S resin. This resin consists of 10% cross-linked styrene-divinyl benzene beads with an average particle diameter of 20 microns. The column is placed in a glass heating jacket in which the circulating water is kept at a uniform temperature with a Haake Type F constant temperature circulator. Another Haake circulator is employed in the reaction bath. A Gilford 2000 spectrophotometer with a 5 mm. flow-through cuvette is used for all color recognition and recording. Teflon spaghetti tubing (20 meters long) is incorporated in the system from the column exit to the cuvette exit in order to prevent mixing which occurs in larger diameter tubing. All tubing connections are stainless steel and were obtained from Swagelok. The eluant and dye are pumped with Beckman Accu-Flo piston pumps (maximum pumping rate of 3 ml/min).

b. Chemicals

Sugars for the preparation of standard solutions were obtained from Fisher Scientific and Eastman Organic Chemicals, and are of reagent grade (> 95% purity).

The tetrazolium'blue' reagent consists of 2.0 grams of p-anisyltetrazolium chloride (obtained from K and K Laboratories) dissolved in one liter of 0.18 molar sodium hydroxide. The reagent is kept in a light-proof aspirator bottle with a delivery stopcock and, when not in use, is stored at 4°C in the dark.

Sodium sulfate, analytical reagent grade, 0.50M, is used to regenerate the column after every ten analyses.

The eluant is made by diluting 95% ethanol (not denatured) to 89% with distilled water.

The distilled water was tested for its sugar content. 1000 milliliters were evaporated under reduced pressure to 1 milliliter and an aliquot was analyzed; no sugars were detected.

c. Procedure

The column was filled by injecting a slurry of the resin into the top of the column. The resin was allowed to settle overnight. Additional resin was added the next day and then, connecting the eluant pump to the column, the bed was packed at the flow rate and temperature to be

used during analyses. The resin, initially in the borate form, was converted to the sulfate form by pumping 200 ml. of 0.5 M Na_2 SO_4 through the resin bed. The column was washed with water and conditioned overnight with the eluant. The column was then ready for chromatographic analyses. Packing, conversion, washing, regeneration and analysis were conducted at 76°C. The resin bed was recessed 14 centimeters in the heating jacket in order to preheat the eluant to the temperature of the column.

Eight monosaccharides were dissolved in water to make a standard solution (1000 ug/ml of each): desoxyribose (d-Ri), rhamnose (Rh), ribose (Ri), arabinose (A), xylose (X), mannose (M), galactose (Ga), and glucose (Gl). The standard was made 10% in isopropanol and stored at 4°C in order to prevent bacterial degradation.

Aliquots of the standard were placed on the column with a microsyringe and forced into the resin bed with 40 to 50 p.s.i. nitrogen. The tubing wall above the resin bed was washed with ethanol which was also forced into the resin with nitrogen pressure. The space above the resin bed was filled with 89% ethanol and the eluant pump (set at 0.5 ml/min) was connected to the top of the column. The dye pump (set at 0.2 ml/min) was connected to the column eluate, and the recorder was switched on.

d. Standardization

The elution order of the monosaccharides was determined by spiking one sugar at a time into the standard. The retention time of each peak relative to desoxyribose was measured and found to be reproducible (Table 1). Quantitative calibration of the system was achieved by use of peak area. Aliquots of the standard (5, 10, 15, 20, 25 ul) were analyzed. Figure 5 depicts the relationship between peak area (measured as the product between the peak height and the width at half height) and the amount of sugar represented by each peak.

Peak height was also found to be proportional to sugar concentration. However, the use of peak volume is more desirable since most sugars react on nearly a mole for mole basis with the reagent, thereby, yielding almost identical peak areas when equal moles of sugars are analyzed. Therefore, by using an internal standard (i.e., addition of a sugar not present in the sample), most peaks present on the chromatogram are quantified by comparison with the peak area of the internal standard. In addition, difficulties in reproducibility arising from fluctuations in temperature, flow rate, ethanol concentration, and dye efficacy are overcome by the use of an internal standard.

2. Construction of Sugar Analyzer: Discussion

The system as depicted in Figure 4 is described according to its two major components: the column and the dye reagent.

a. Column

The following parameters had to be adjusted during the construction of the sugar analyzer: 1) type of resin; 2) length and diameter of resin bed; 3) column temperature; 4) type of eluant; 5) pumping mechanism; and 6) eluant pumping rate.

These variables were found to be interdependent and, therefore, in order to examine the effect of any one of them on the resolution and sensitivity, it was necessary to hold the others constant.

1) Resins

Columns can be packed with various materials. Activated carbon, Fuller's earth clay, carbohydrates, ion-exchange resins, and calcium acid silicate have been employed with varying success to separate sugars (Lederer and Lederer, 1955). Of these, ion-exchange resins were found to yield excellent separations through partition chromatography and, therefore, are employed in the present analytical system.

Strongly cationic and anionic exchange resins have been employed in sugar analyzers described in the literature.

For example, anion exchanger Dowex 21K (1 to 16 microns) in the chlorinated and sulfonated forms (Larsson and Samuelson, 1965) and cation exchanger Dowex 50 W-X8 (14 to 17 microns) in the potassium, sodium and lithium forms (Samuelson and Strömberg, 1968) have been successfully applied. Any strong ion-exchange resin can be used, however, the success of separation is dependent upon the particle size and sorting (Samuelson, written communication, 1969). Examination of Figure 6 reveals that fine-grained resins of uniform particle size give the best resolution. For this reason, the Technicon resin (Figure 6) was found to be superior to Dowex 21K (37 -74 μ), Dowex 50W - X8 (37 - 74 μ), and Dowex 1 - X8 (37 - 74 μ).

2) Length and Diameter of Resin Bed

Columns of various dimensions were packed with the Technicon resin. Qualitatively, longer resin beds yielded the greatest resolution while narrower resin beds yielded the greatest sensitivity. Since low sugar concentrations are being examined, a long (110 cm.) resin bed having a narrow (0.28 cm.) diameter is employed.

3) Column Temperature

Peaks became sharper and better resolved at elevated temperatures. Figure 7 illustrates this effect. Chromatogram A was run at 60°C, and B at 76°C. At 60°C, galactose

and glucose are poorly resolved, while at 76°C they are completely separated. The effect of elevated temperature is due to an increased rate of diffusion inside the resin particles, which, in turn, reduces both the time for analysis and the pressure drop across the column (at eluant flow rates of 0.5 ml/min., pressures of 400 to 500 p.s.i. were encountered at 76°C, while pressures of 600 to 700 p.s.i. were measured at 60°C).

4) Type and Concentration of Eluant

Two elution methods are commonly used: NaCl - borate eluant; and ethanol - water eluant. In the first method, sugars interact with the borate ion to form negatively charged complexes. The separation of the sugar - borate species arises from electrical interaction between these ion complexes and strongly anionic resins (Kesler, 1967).

In the second method, the separation of sugars is due to an uneven distribution of water between the resin and mobile phases. The amount of water in the resin is higher than in the external solution and since sugars are polar, they are held more strongly by the resin phase. In general, the more polar groups contained in a sugar molecule, the stronger it is held by the resin. Therefore, the general order of elution is: pentoses, hexoses, disaccharides, trisaccharides, etc. (Arwidi and Samuelson, 1965).

Both the NaCl-borate and ethanol-water elution techniques yield excellent sugar separations. However, the ethanol eluant was chosen because the kinetics of the tetrazolium 'blue'-sugar reaction were found to be extremely sensitive to the high concentrations of dissolved salts present in the NaCl-borate eluants.

The effect of varying the ethanol concentratration was determined. High ethanol concentrations (> 92%) led to increased retention times, large elution volumes, peak broadening, and excessive separations. The analysis depicted in Figure 8, which was run with 92% ethanol, was completed in three and a half hours. Chromatogram B in Figure 7 (89% ethanol) was completed within two hours. At concentrations under 85% ethanol, peaks were insufficiently resolved to allow for quantitative evaluation. Therefore, the working ethanol concentration represents a compromise between the degree of separation and the time for analysis. The ethanol concentration presently being used is 89%.

5) Pumping Mechanisms

Both the eluant and the dye are fed into the system with piston pumps. However, initially, the dye was forced into the system with nitrogen pressure. This technique was found unsatisfactory because fluctuations in the 'tank' pressure (from 1. minor leaks in the nitrogen line;

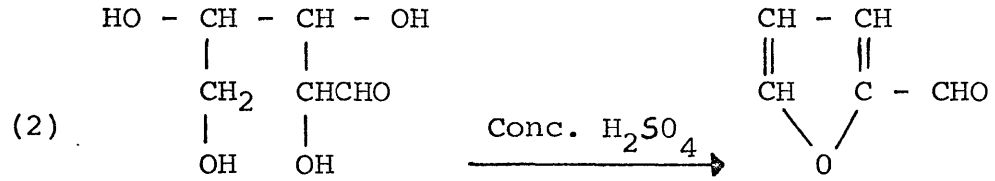
and 2. lowering of the dye reservoir) resulted in erratic baselines and poor reproducibility of peak areas.

6) Eluant Flow Rate

High flow rates led to decreased elution times and poor resolution. Figure 9 illustrates these effects. Xylose and mannose in chromatogram A are non-resolvable at a flow rate of 1.2 ml/min. However, these peaks are well separated in chromatogram B which was run at 0.6 ml/min. In addition, higher pressure drops across the column were measured at the faster flow rate placing considerable strain on the column tubing. At a flow rate of 1.5 ml/min, one column burst (76°C, 800 p.s.i.).

b. Dye Reagent

Insensitivity and corrosiveness of the dye reagents are major difficulties in automatic sugar analyzers built by other investigators. These dyes include: orcinol-sulfuric acid (Arwidi and Samuelson, 1965), anthrone-sulfuric acid (Dregwood, 1946), phenol-sulfuric acid (Dubois et al., 1956 and Handa, 1966), n-ethylcarbazole-sulfuric acid (Zein-Eldin and May, 1958) and cysteine-sulfuric acid (Dische, 1949). The stain mechanism depends on the dehydration of sugar by concentrated sulfuric acid to form furfural and a large number of intermediate products related to, or derived from furfural (Fieser and Fieser, 1961):



sugar furfural

The furfural and the intermediary products (i.e., furfural alcohol) react with the dye, to form colored complexes. However, the nature, and hence the reactivity, of the intermediary products varies with each sugar being analyzed (Dische, 1950). Therefore, individual calibration curves must be constructed.

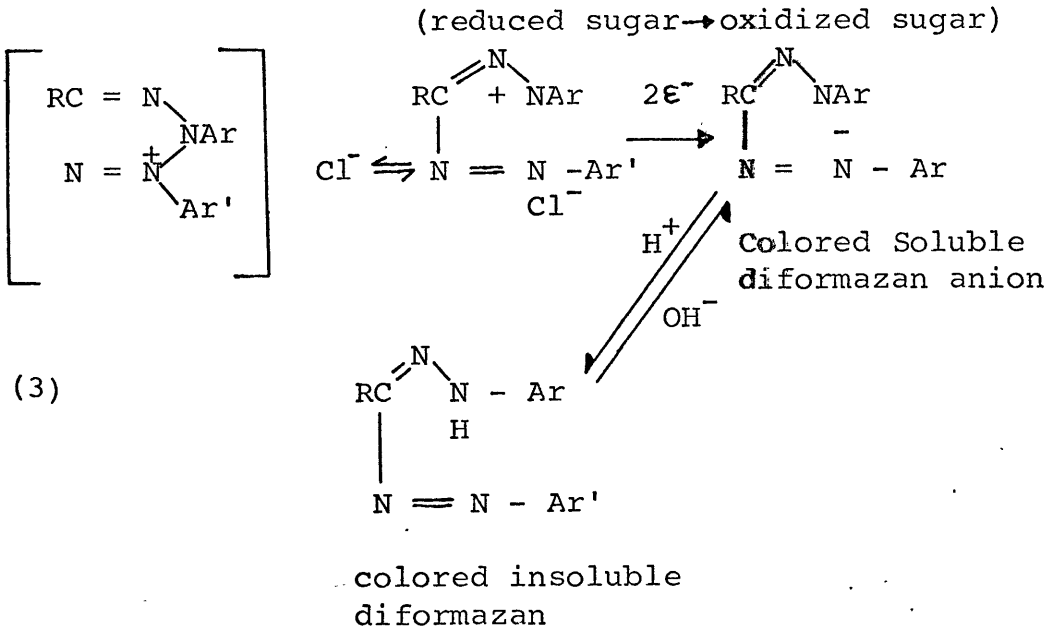
In addition, because of the varying reactivities of the intermediary products of sugar dehydration, the intensity of the final solution is lower than if only furfural were reacting. The limit of detectability of these stains is 10^{-7} - 10^{-8} moles.

These difficulties are avoided in the present system by employing a uniquely sensitive, and relatively non-corrosive dye: p-anisyl tetrazolium chloride (or tetrazolium 'blue'). Up till now, this dye has not been used in automatic sugar analyzers because of the precipitation of diformazan (reaction 3) which would clog the capillary tubing. However, through extensive laboratory tests by the author, the problem of the precipitation was solved, thereby, rendering tetrazolium 'blue' applicable to the automatic

sugar analyzer.

Work on the reaction kinetics between tetrazolium salts and oxidizable organic compounds, such as sugars, was done by Cheronis and Zymaris (1957). However, their results were intended to apply to total sugar analyses of blood samples and, therefore, could not be directly incorporated into the sugar analyzer because of the precipitation of diformazan (reaction 3).

(3)



In the procedure of Cheronis and Zymaris (op. cit.), this problem is overcome by dissolving the precipitate in dioxane or acetone. Their procedure is as follows: 0.1 ml. of a 1.0% solution of tetrazolium 'blue' in ethanol, 0.3 ml. of 0.3 M NaOH, and aliquots of sample were pipetted into test tubes (19 x 150 mm). Water was added to bring the final volume up to 1 ml. The mixture was heated at its boiling

point for one minute and cooled in a 20°C water bath for three minutes. The diformazan precipitate was dissolved by the addition of 9 ml. of either dioxane or acetone. The absorbance was determined spectrophotometrically at 615 mμ; the color was stable for at least an hour.

The above precedure has been adapted to the analysis of the total sugar content of natural samples; however, in order to use this reagent in the automatic sugar analyzer it was necessary to prevent the diformazan from precipitating.

Dioxane and acetone were partially substituted for water in the procedure of Chernois and Zymaris (op. cit.). It was thought that these solvents would dissolve the precipitate while it formed. However, blank analyses (absence of sugars) indicated that both dioxane and acetone greatly upset the reaction kinetics causing large quantities of diformazan to precipitate during the boiling step.

When ethanol (95%) was substituted for water in the procedure, blank analyses indicated that: 1) only the soluble diformazan anion formed (reaction 3); and 2) when the reaction mixture contained 60% (or less) ethanol, only a faint pink coloration resulted. Aliquots of a standard glucose solution ranging from 0.05 μg. to 70 μg. were analyzed in 1 ml. of the reagent solution containing 60%

ethanol. The results are plotted in Figure 10. This figure indicates that if the sugar concentration is below 50 ug/ml, diformazan precipitation does not occur.

It was mentioned in the standardization section that most sugars appeared to react on a mole for mole basis with the tetrazolium reagent. This equivalency was also observed by Cheronis and Zymaris (op. cit.) who determined that 2.78×10^{-7} moles of glucose precipitated 2.68×10^{-7} moles of diformazan from a standard tetrazolium 'blue' solution. The basis for this equivalency is evident in reaction (3). For every mole of sugar oxidized, a mole of tetrazolium 'blue' is reduced. Therefore, by using an internal standard, most peaks in a chromatogram can be quantified. This contrasts with other stains where each sugar reacts differently with the dye reagent.

In addition, the tetrazolium dye is slightly basic (pH = 12.5) and, therefore, is non-corrosive (relative to concentrated sulfuric acid). The reagent is pumped with a normal piston-type pump employing steel connections.

All absorbances are being read at 520 mu. Figure 11 shows the absorbances of a standard sugar solution at different wavelengths.

IV CONCLUSIONS AND PROSPECTUS

An automatic sugar analyzer has been constructed which is rapid (3-4 hours), highly sensitive (10^{-10} moles), reproducible, and which yields high resolution.

Improvements in the system are being initiated to:

1) Increase the resolution; construction of columns 160 cm. long assures the complete separation of all monosaccharides present in natural samples as well as laboratory standards;

2) increase the output; by constructing two additional columns, three samples can be analyzed simultaneously (and a total of six a day);

3) automatically integrate and digitize the peak areas; this will facilitate the quantification of large amounts of data; Figure 12 depicts a chromatogram which was digitized (numbers inside peaks) by a Dual-Channel Digital Integrator and Printer (Infotronics, Inc.); the instrument is an integral part of an automatic amino acid analyzer built by Egon T. Degens in our laboratory.

PART TWO

I. EXTRACTION OF SUGARS FROM SEDIMENT AND SEAWATER — FIRST ANALYSES

INTRODUCTION

Extraction of sugars from natural samples is difficult because the procedures previously employed by other researchers result in the alteration and destruction of a fraction of the released sugars. Therefore, prior to analyzing carbohydrates from the marine environment, it was necessary to determine very precisely the effects of the extraction procedures on standard solutions. Then, sugar losses or alterations occurring in the extraction of the natural samples can be calibrated with the amount of loss of the standard solutions.

1. Extraction of Soluble Sugar

a. From Water:

Figure 13 is a flow diagram depicting the following procedures for the extraction of soluble sugars from salt water. The initial volume of the sample (one to ten liters) is measured accurately. The sample is then suction-filtered through glass fiber filter pads (pore size, 0.3 microns). Particulate matter is stored at 4°C awaiting further treatment. The filtrate is reduced to 20-30 ml. in a flask evaporator and then split. One half is analyzed for monosaccharides; the other half is hydrolyzed in order to convert the soluble oligosaccharides and polysaccharides to their monomeric constituents. Hydrolysis is achieved by refluxing the sample in 0.5 N H_2SO_4 for eight hours under a blanket of nitrogen. The H_2SO_4 is neutralized by $BaCO_3$. With the addition of $BaCO_3$ large quantities of $BaSO_4$ precipitate formed. It was thought that this precipitation might have induced a partial "co-precipitation" of some sugars (Jeffrey and Hood, 1958; Bader et al., 1960). To test this, the precipitation was carried out with 1 ml. of a standard solution of eight monosaccharides (1000 ug/ml of each). The precipitate was filtered out of the solution and washed thoroughly with 20 ml. of hot 70% aqueous ethanol. The filtrate and washings were then combined and reduced to a standard

volume and analyzed. All sugars were quantitatively recovered with the exception of desoxyribose which was reduced by 40%. The loss of this sugar, however, was undoubtedly due to the acidification step prior to neutralization and not to a "co-precipitation" effect. Further evidence for the frailty of this sugar in acid solutions will be presented later in this thesis.

Desalting of the samples (both the hydrolyzed and unhydrolyzed fractions) is accomplished in three connected ion-exchange columns. The upper and lower columns contain AG50W-X8, a strong cation exchange resin (H^+ form). The middle column contains AG3-X4, a weak anion exchange resin (OH^- form). Both resins were obtained from Bio-Rad Inc. The upper cation and middle anion columns function to deionize the sample, while the lower cation column serves to modify the pH (final eluate pH=5). The pH control is essential since the eluate from the anion exchange column is mildly alkaline, a condition which can cause sugar isomerization, fragmentation and intra-molecular oxidations and reductions.

This method of desalting removes all basic and acidic organic molecules such as amino acids, carboxylic acids, and amino sugars. Monosaccharides are neutral and, therefore, are not held by the exchange resins, although they

may be altered in the anion exchange column. Therefore, tests were conducted to determine the effects of ion-exchange resins on standard sugar solutions. For example, 1 ml. of an eight-component standard solution (1000 ug/ml of sugar) was placed on the first ion-exchange column and eluted with 300 ml. of H_2O. This test was then repeated using 300 ml. of 70% ethanol as the eluant. The results are tabulated in Table 2. In both tests, all the sugars were recovered quantitatively (±10%) with the exception of desoxyribose. This loss is readily apparent in the chromatograms reproduced in Figures 14 and 15. The low recovery of desoxyribose was again due to its fragility in acidic solutions.

To test whether new sugars were being produced by molecular re-arrangement of desoxyribose on the resins, a standard solution consisting only of desoxyribose and ribose was placed on the ion-exchange columns. The result is illustrated in Figure 16. Fifteen percent of the ribose was lost and ninety percent of the desoxyribose was destroyed. Trace amounts of five other sugars were formed in this experiment. However, the amounts produced do not nearly equal the amount of desoxyribose that was lost. Therefore, other processes are responsible for the destruction of desoxyribose.

IR analyses of pure desoxyribose and 'desoxyribose' that was passed through ion-exchange columns are shown in Figures 17 and 18 respectively. The IR chromatogram of pure desoxyribose is identical to that of other monosaccharides with the exception of the absorbance peak at 1450 cm^{-1} which is characteristic of methylene ($>CH_2$) bending. The chromatogram of the resin-treated 'desoxyribose' clearly indicates that the original molecule is totally destroyed (fragmented). Further tests are being conducted to verify these data.

Destruction of desoxyribose may be prevented by buffering the exchange resins by keeping pH slightly acidic. Ammonium bicarbonate is a desirable buffer because all traces of this compound can be removed during flash evaporation of the eluate.

To quantitatively isolate dissolved organic compounds from sea water is exceedingly difficult. Several methods, in addition to ion-exchange, considered during this study were: dialysis, electrodialysis, column adsorption, solvent extraction, and co-precipitation with iron (III) hydroxide. A detailed description of these methods is given in Jeffrey and Hood (1958). Several difficulties are common to all these techniques. For example, the ratio of

dissolved organic matter to inorganic salts in seawater is approximately 10^{-4}; in other words, 3 to 4 mg/l of organics must be separated from 35,000 mg/l of salts. In addition, sugars (as well as other organic nutrients) are subject to: 1) bacterial attack; 2) destruction or alteration by heat; and 3) modification by change in the chemical environment. The loss of desoxyribose on ion-exchange resins is a relevant example of this labileness. Furthermore, some organic compounds strongly adsorb to glass or polyethylene. Methods which treat these problems must invariably involve a compromise between final salinity and organic yield.

Of the desalting techniques listed above only column adsorption and solvent extraction appear acceptable. Several adsorbents have been tested and their capabilities to attract carbon-14-labelled organic compounds dissolved in sea water have been measured (Jeffrey and Hood, 1958): activated carbon: 100% adsorption; acid alumina, 65%; silica gel, 15%; and calcium carbonate, 50%. Activated carbon is the most efficient adsorbent. A sample can be rapidly desalted by simply filtering it through a column of this material followed by washings with distilled water. However, several difficulties are inherent in this technique. For instance, many amino acids are so strongly adsorbed that no effective method has been found to desorb

them (Jeffrey and Hood, 1958). Sugars, however, are readily recovered by elution with aqueous ethanol (Whistler and Durso, 1950).

Organic solvents which can be employed to extract sugars from sea water are: n-butanol and pyridine. N-butanol is used in a liquid-liquid extraction technique. Water is only partially soluble in n-butanol so that when they are mixed two phases form. One phase will be predominantly organic, the other aqueous. The sugars, being organic in nature, will be more soluble in the organic phase than in the aqueous phase. The salts, however, being more hydrophilic than sugars, will remain in the aqueous phase (Boylan, pers. comm., 1970). Several extractions will remove most of the sugars to the n-butanol phase. The combined n-butanol extracts can then be back-extracted with pure water to remove any remaining salts. The water from this extraction is then back-extracted with n-butanol to remove any sugars which might have dissolved in the water.

A somewhat simpler method involves the use of pyridine. A salt water sample is reduced to dryness in a flash evaporator. The residue is then treated for several minutes with hot pyridine. The resulting solution is cooled, filtered, and evaporated to dryness. This residue is then re-extracted with pyridine to lower further the salt concentration.

b. From Sediment :

Figure 19 is a flow diagram depicting the extraction procedure for removing soluble sugars from sediments. Ten to fifteen grams of wet sediment are weighed into a 125 ml. Erlenmeyer flask (24/40 standard taper neck). Twenty-five milliliters of 70% aqueous ethanol are added. The sample is dispersed with a magnetic stirrer and then refluxed for 16 hours under a blanket of nitrogen. The sample is then transferred to centrifuge tubes and centrifuged at 10,000 r.p.m. for 8 minutes. The extract liquid and washings are combined and suction-filtered through 0.3 micron glass fiber filter pads. The volume is reduced to 10 ml. and deionized or ion-exchange resins. The eluate is concentrated in a flash evaporator to a precise volume and then analyzed.

Initially two methods for extracting the sugar, one using water and one involving 70% aqueous ethanol, were used. A Black Sea core sample was first extracted with water and then with 70% ethanol. Chromatograms of the two extracts are reproduced in Figures 20 and 21. It is apparent from these chromatograms that the ethanol extraction procedure is considerably more efficient.

Further tests were conducted to determine if ethanol extractions were 1) effectively removing all soluble sugars

adsorbed onto clays and 2) causing selective destruction and/or synthesis of sugars. Sediment from a Black Sea core was ignited at 800°C for three hours to remove all organic compounds. One milliliter of a standard solution of eight monosaccharides (1000 µg/ml of each) was added to two grams of the residue. The slurry was stored at 4°C for three days and then refluxed with 70% ethanol. The resulting chromatogram is shown as Figure 22. All sugars were recovered quantitatively (±10%) with the exception of desoxyribose (30% lost). In addition, two new sugars were synthesized in trace quantities. The peak (Figure 22) between arabinose and ribose has been tentatively identified as lyxose. The peak preceding desoxyribose has not been identified. The process resulting in the synthesis of these sugars is not yet understood. However, minerals (i.e., clays) in the ignited sediment might provide active catalytic surfaces for intra-molecular rearrangement of some of the original sugars. (Studies on this process are underway in our laboratory.)

2. Soluble Sugars - Discussion and Preliminary Analyses

Although soluble sugars can be readily extracted from organisms, sea water, and sediment pore water, very little work has been done on enumerating the types and quantities of sugars present in these systems, therefore,

the exact nature of the interrelationships and fractionations of soluble sugars in the marine environment is largely unknown. However, preliminary laboratory work by Bristol-Roach (1928), Lewin and Lewin (1960), Taylor (1960a,b) and Allen White (pers. comm., 1970) indicates that the growth of certain plankters is directly related to the glucose concentration in the culture media and that these organisms preferentially absorb this sugar in the presence of other food sources.

It is also known that plankton can influence the distribution and concentration of soluble sugars in natural environments. For example, Okaichi (1967) was able to correlate Red tides in the Seto Inland Sea with the concentrations of total dissolved carbohydrates. He found that under normal conditions the surface waters of the Seto Inland Sea contained 0.5 mg/l dissolved carbohydrates; while during Red tides as much as 1.2 - 6.8 mg/l were present. In addition, seasonal and diurnal fluctuations in the total dissolved carbohydrates have been observed by several investigators (Walsh, 1965a,b; 1966: Sugawara, 1965; Sprinivasagan, 1965; Walsh and Douglass, 1966; Semenov and Vlasova, 1967; Semenov and Ptskialadze, 1968; and Handa, 1967). For instance, Walsh (1966) discovered seasonal fluctuations of dissolved carbohydrate in Oyster Pond,

Massachusetts. He observed that in February, when biological activity was at a minimum, total dissolved carbohydrates was approximately 1.3 mg/l; while in April during a spring algal bloom, he recorded a value of 2.9 mg/l. All Walsh's quantitative determinations were achieved with the anthrone-sulfuric acid dye technique. This reagent, as pointed out by Hoffpauir (1952), is not specific for sugars, since dissolved proteins and organic acids will also react to produce colored compounds. In addition, inorganic salts can interfere with the determination. Therefore, the data measured by Walsh (op. cit.) should be considered only 'qualitatively' correct.

No detailed analyses of the monosaccharide composition of dissolved carbohydrates in lake or ocean waters exists in the chemical literature (with the exception of Vallentyne and Whittaker, 1956). Only total dissolved sugars have been measured and many of these analyses involved poor analytical techniques.

Therefore, a detailed qualitative and quantitative study of dissolved carbohydrates in water from various environments is long overdue. Environments that should be examined include: open ocean, anoxic basins, polluted and unpolluted estuaries, rivers, lakes, and salt marshes. Factors which may control the monosaccharide composition of

water in these environments are: 1) the composition of the source material; 2) the productivity of the waters; 3) the susceptibility of the compounds to microbiological degradation; and 4) sample depth in the water column.

Such a study would be useful from a biological standpoint, since many species apparently are influenced by, and may indeed depend upon the sugar concentrations in their micro-environments (White, pers. comm., 1970).

Research on the waste effluent of the Revere Sugar Company in Boston, Massachusetts is now being initiated in our laboratory. This plant dumps enormous quantities of dissolved sugars into adjacent rivers and in doing so completely disregards the effects of its effluent on the ecological cycles in the river systems. These effects will be examined.

The distribution of soluble sugars in sediments has also received little attention since researchers have a priori believed that high bacterial concentrations at the sediment-water interface would effectively remove all labile nutrients, such as free sugars (Vallentyne, 1963). However, studies by Vallentyne and Bidwell (1956), Whittaker and Vallentyne (1957), and Abrosimov and Kornilova (1967) have documented the presence of free sugars in sediments of fresh water lakes. Abrosimov and Kornilova

(op. cit.) only determined total soluble sugar concentrations. The studies of Vallentyne and Bidwell (op. cit.) and Whittaker and Vallentyne (op. cit.) however analyzed the individual monosaccharides and oligosaccharides and found that maltose and glucose were the dominant sugars; sucrose, fructose, galactose, arabinose, xylose, and ribose were present in trace amounts. However, since these studies contained noteworthy experimental errors (i.e., 1. 60 - 90% ribose, xylose, glucose, and galactose were lost during the desalting process; and 2. the sugars were separated and detected on paper chromatograms, a method with an inherent 20 - 30% error), these results can only be considered preliminary.

Up till now only one analysis has been reported on the distribution of free sugars in oceanic sediments (Plunkett, 1957). The analysis was only qualitative; sucrose, glucose, fructose, galactose, arabinose, and xylose were identified. The present investigator has analyzed sediment from a Black Sea core for free sugars. The chromatogram is shown in Figure 21. By comparing my data with that of Whittaker and Vallentyne (op. cit.), one can readily observe quantitative and qualitative differences. In the Black Sea sediment, the pentoses are as important (if not more so) than the hexoses; while in

lake sediments (Whittaker and Vallentyne, op. cit.), the reverse is true.

The studies described above suggest that the types and quantities of free sugars found in sediment are controlled by their environment of deposition. Work is presently being conducted on sediment from several different environments, i.e., open ocean, closed basins, polluted estuaries, unpolluted estuaries, etc. to test this idea.

Degens (1967) has postulated that free organic compounds migrate in the sediment column and become separated by chromatographic processes. His hypothesis stems from the discovery that there is a separation of amino acids in the sediment of Santa Barbara Basin off California. A study is being undertaken to test his hypothesis on sediments from the Black Sea which, like Santa Barbara is an anoxic basin. The primary attribute of an anoxic basin which recommends it to this type of study is that organic debris accumulate undisturbed in the sediments. This is due to the fact that below a certain depth in the water column the concentration of dissolved oxygen falls to zero. Therefore, with the exception of hydrogen sulfide-producing anaerobes, the basin sediments are abotic. (For a full discussion of the preservation of organic matter in anoxic marine environments see Richards, 1968). Under these

circumstances free organic molecules, such as sugars and amino acids, will migrate upward in the interstitial water during diagenesis. By this process, free organic molecules may become selectively adsorbed and desorbed on clay particles. A chromatographic separation of the organics may result. Degens (op. cit.) demonstrated this process for the amino acids in the Santa Barbara Basin sediments (see Figure 23).

Figure 24 includes relevant information on Black Sea core #1474K. From this figure, it can be seen that the segment between 30 and 70 cm. contains approximately 15% organic carbon. This part of the core is being analyzed in great detail in hopes of detecting a chromatographic separation of the free sugars.

One additional problem remains to be solved: the origin of free sugars in sediments. Three sources are being considered: 1) gradual depolymerization of polysaccharides; 2) excretions released from living plants and animals; and 3) enzymatic hydrolysis of polysaccharides by bacteria. The latter mechanism was proposed by Pochon and Chalvignac (1951) for the breakdown of starch in soil. The relative importance of each of these mechanisms will no doubt vary with the depositional environment.

III INSOLUBLE SUGARS

1. Hydrolysis Procedures

Two types of material are of interest: particulate organic matter in sea water (seston) and residual carbohydrates in sediments after extraction with aqueous ethanol. Several methods have been described in biochemical literature for the depolymerization of polysaccharides (i.e., Waksman and Stevens, 1928; Heuser, 1944; and Whistler and Smart, 1953). These methods rely on the action of dilute mineral acids to hydrolyze the glycoside bond. Unfortunately none of these methods can totally depolymerize all the polysaccharides present in the sample yet not destroy to some extent the fragile monomeric sugar units. In other words, if the acid concentration is too dilute only partial hydrolysis will occur. If the acid concentration is too strong molecular-rearrangement and dehydration of monomeric sugars will occur (see reaction 2).

The concentration of acid used will depend upon the sample being examined. For example, if the sample consists essentially of mannans (i.e., seaweeds), then refluxing for 8 to 10 hours in 0.1 N H_2SO_4 will be sufficient. However, if the sample consists mostly of cellulose, a more highly cross-linked polysaccharide, 1.0 N H_2SO_4 will be needed. The latter procedure is as follows (Degens and Reuter, 1964):

the sample (10 to 20 mg. of seston or 5 to 10 g. of sediment) is cooled to 4°C; 0.5 to 2.0 ml. of pre-cooled H_2SO_4 (98%) is added. The sample is allowed to sit in the concentrated acid for two hours at 4°C. This treatment swells the lattices of even the most resistant polysaccharides and renders them soluble in dilute acid. Ice is then added until a final concentration of 1 N is reached. The sample is refluxed under an atmosphere of pure nitrogen for eight hours. The sample is then centrifuged at 10,000 r.p.m. for 8 minutes. The hydrolysis liquid is decanted and the residue is thoroughly washed with hot distilled water and then re-centrifuged. The hydrolysis liquid and washings are combined. The H_2SO_4 is removed by neutralization with $BaCO_3$ as described previously. Inorganic salts and charged organic compounds (i.e., amino acids and aminosugars) are removed on ion-exchange resins. The eluate is reduced to an exact volume and analyzed.

2. Tests of Hydrolysis Procedures

Figure 25 depicts a chromatogram of a standard sugar solution of eight monosaccharides (1000 µg/ml of each) of which 1 ml had been 'hydrolyzed' as described above. By comparing the chromatogram with that of the untreated standard solution (Figure 14), it is apparent that only desoxyribose was affected by the procedure. Since it was

demonstrated earlier that this compound is labile in even mildly acidic solutions, this result was expected.

1 ml. of the same standard solution was again 'hydrolyzed' as above, however, this time, two grams of ignited Black Sea sediment (800°C for three hours) were added. The results are shown in Figure 26. Comparison of this chromatogram with that of the untreated standard (Figure 14) demonstrates that all sugars were destroyed to varying degrees. The losses are tabulated in Table 3.

It was previously hypothesized that catalysis on mineral surfaces was active in the formation of new sugars from pre-existing sugars while refluxed in 70% ethanol (Figure 22). Therefore, it is reasonable to believe that minerals in the ignited residue were also active in catalyzing the destruction of the sugars during acid 'hydrolysis'. Further work is being conducted to verify this.

HCl was also tested in the hydrolysis procedure. This acid would be desirable to use since, after hydrolysis, most of the HCl can be evaporated under vacuum, thereby, avoiding the tedious process of neutralization (Petersson et al., 1969). The procedure outlined by Tracey (1955) for the hydrolysis of chitin was examined. In his procedure, the sample was treated with 6 N HCl for six hours at 100°C. One milliliter of an eight component standard sugar solution

(1000 µg/ml of each sugar) was used to test the procedure. The results are indicated in Figure 27. Comparison of this chromatogram with that of the untreated standard (Figure 14), clearly indicates that all the sugars were > 95% destroyed during the 'hydrolysis'. The analysis was repeated with 1.5 N HCl. The results are shown in Figure 28. The chromatogram indicates that sugars are also sensitive to dilute HCl. Furthermore, a "hydrolysis" test with 1.5 N HCl in the presence of two grams of ignited Black Sea sediment (800°C for three hours) resulted in even further losses.

The fractional losses from the hydrolysis tests mentioned above have been tabulated in Table 3. Sulfuric acid is safer than hydrochloric acid for the hydrolysis of clay-free carbohydrate material (i.e., particulate organic matter in sea water). However, for analyses of sediment, neither technique will yield a true representation of the monosaccharide composition present. Variability in the size and composition of the mineral fraction in sediments will result in variability in the fractional losses of each sugar during acid hydrolysis.

Other hydrolysis techniques are under examination. These include the use of 1) strong cation exchange resins in the hydrogen form (Abdel-Akher, 1958); 2) dilute mineral acids such as $HClO_4$, BCl, and BF; and 3) enzymes.

3. Discussion

Despite (or perhaps ignorant of) the difficulties discussed in the previous section, several investigators have extensively examined sugars released from rocks and sediments by acid hydrolysis (Prashnowsky, et al., 1961; Swain, 1966; Swain et al., 1967; Havrankova, 1967; Formina and Nappa, 1967a,b; Prashnowsky and Schidlowski, 1967; and Swain, 1969). For example, Swain et al., (op. cit.) hydrolyzed a variety of rock types from different geological periods with dilute H_2SO_4. Based on these analyses, they made interpretations about paleoenvironments. These interpretations were based upon three assumptions: 1) the qualitative and quantitative composition of polysaccharides in the sediments from different environments is variable; 2) lithification of the sediments did not affect the more resistant polysaccharides; and 3) all polysaccharides are extracted quantitatively from the samples.

The first assumption is probably correct. Since, as was demonstrated earlier, the composition of the soluble sugars in sediments is influenced by the depositional environment, it is reasonable to believe that the composition of the insoluble sugars will also be so influenced. The degree to which the second assumption is correct depends upon various factors: 1) the geological history of the rock

(i.e., extent of metamorphic alteration by heat and pressure, periods of folding and faulting, etc.); and 2) the extent to which the rock has remained a closed system (i.e., its permeability to percolating solutions). Therefore, rock samples which have experienced only mild geological and chemical metamorphism will contain approximately their original polysaccharide composition. The third assumption, however, does not hold up. It was demonstrated in the previous section that hydrolysis of a standard sugar solution with dilute H_2SO_4 in the presence of minerals (i.e., clays) resulted in the partial destruction of most of the monosaccharides (Figure 26). Swain et al. (1967) noted that " the sum of the monosaccharides extractable by hydrolyzing the [rock] sample with weak acid is much lower than the total carbohydrates extracted in the form of furfural with strong acid." Therefore, until polysaccharide extraction procedures have been greatly improved, continued analysis of sediments and rocks will yield only partial and biased results. Conclusions drawn from these results will, therefore, be inaccurate.

From an analytical viewpoint, the analysis of particulate organic matter (and other clay-free substances) is more rewarding. Particulate organic matter, also known as seston, consists of living organism (phytoplankton, zoo-

plankton, and bacteria) and detritus.

Several total carbohydrate analyses of seston material have appeared in the biological literature (i.e. Birge and Judy, 1926; Raymont and Krishnaswamy, 1960; Raymont and Conover, 1961; and Kostilov, 1966), however, only in a very few instances has the monosaccharide composition of the sestonic carbohydrates been determined (Hough et al., 1952; Whittaker and Vallentyne, 1957; and Parsons and Strickland, 1962). Therefore, detailed carbohydrate analyses of seston from different environments is long overdue. Profiles from the following environments are being considered: open ocean, closed basin, lakes, and polluted bays and estuaries. If the carbohydrate composition varies significantly between these environments, one may, for example, follow the mixing of polluted and unpolluted waters by analyses of the particulate organic matter. This study would be 'relevant' because carbohydrates account for 60-70% of the organic matter in most domestic and industrial wastes (Teletzke, et al., 1967).

IV CONCLUSION and PROSPECTUS

An automatic sugar analyzer, sensitive to 10^{-10} to 10^{-11} moles, based on column chromatography has been constructed in order to examine the distribution of carbohydrates in marine environments.

Procedures were established for extraction of 1) free sugars from seawater, sediments, and seston; and 2) combined sugars from seawater and seston. However, extraction of combined sugars from sediments by dilute acid hydrolosis proved to be exceedingly difficult. Apparently mineral surfaces in the sediment catalyze the destruction and molecular re-arrangement of monosaccharides released during acid hydrolysis of polysaccharides. Enzymatic hydrolysis techniques are being examined as a viable alternative.

Additional evidence for mineral surface-sugar interactions was observed after a slurry of ignited sediment (800°C for three hours) and standard sugar solutions were refluxed for 16 hours. Lyxose and one as yet unidentified sugar were produced by molecular re-arrangement of the pre-existing sugars. Further research on this process is being pursued in our laboratory. In addition, synthesis of sugars from simple organic molecules (e.g. glycerine and formaldehyde) on clays is being attempted.

The first quantitative anlyses for free monosaccharides in Black Sea sediments indicates that these sugars represent several percent of the total organic matter. Furthermore, comparison of these data with analyses of free sugars in lake sediment (Vallentyne and Whittaker, 1957) suggests that depositional environment is definitely linked with the sugar content of the sediment. Therefore, sediments from different environments (open ocean, polluted and unpolluted estuaries, rivers, and salt marshes) are presently being analyzed to test this idea.

Preliminary laboratory experiments indicate that the free sugar concentration of lake and seawater affects the growth of some plankton species. Plankton density in a water body may likewise affect the concentrations of free sugars there. Therefore detailed studies of the sugar content of dissolved and particulate organic matter from several areas of high biologic productivity are being initiated in order to examine such interrelationships.

It is known that carbohydrates account for 60 to 70% of the organic matter in domestic and industrial wastes. The new sugar analyzer system is an ideal apparatus for measuring and identifying the sugar content of polluted areas, such as the effluent of the Revere Sugar Company, Boston, Massachusetts and the sewage disposal area in New York Bight.

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FIGURE 1. G. C. chromatogram of 5 sugars. Sugars were converted to volatile TMS derivatives and separated on a gas chromatograph. Fourteen peaks are present as a result of the detection of α and β anomers of sugar enantiomorphs (Ellis, 1969).

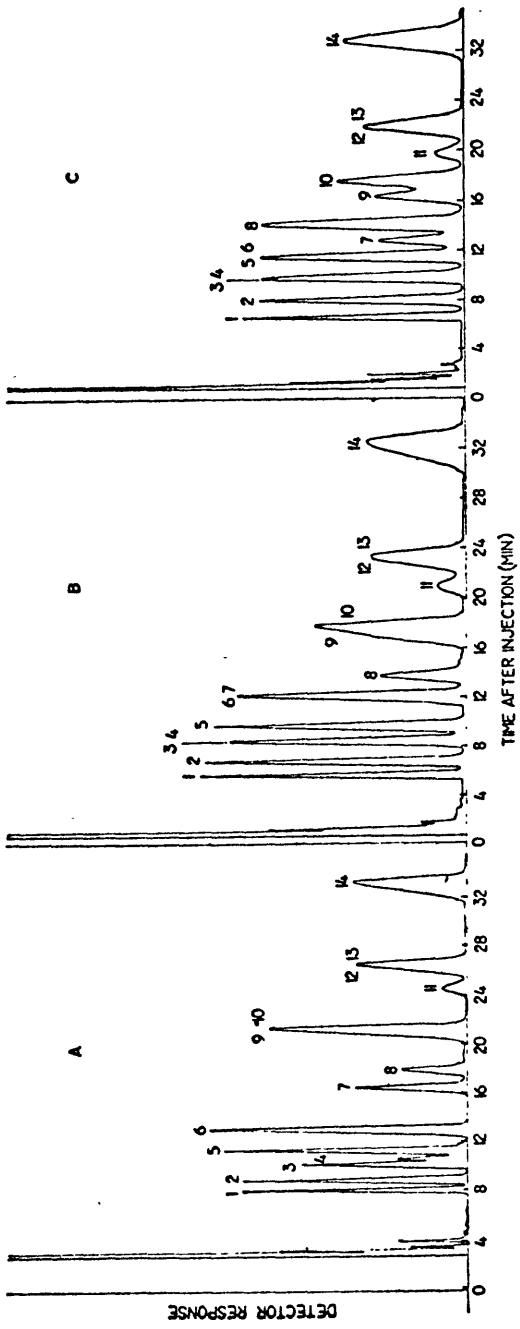


Fig. 1. Separation of a mixture of TMS monosaccharides as influenced by XE-60 concentration on Chromosorb W. Chromatogram A: 1% XE-60, 150°, 11.2 ml N_2/min. Chromatogram B: 4% XE-60, 155°, 12.2 ml N_2/min. Chromatogram C: 16% XE-60, 175°, 18.0 ml N_2/min. TMS arabinose, peaks 1, 2, 3 and 4; TMS xylose, peaks 5 and 6; TMS mannose, peaks 7 and 12; TMS galactose, peaks 8, 9, 11 and 13; TMS glucose, peaks 10 and 14.

FIGURE 2. Mass spectrum of TMS derivatives. Extensive fragmentation of TMS sugar derivatives makes interpretation of their mass spectrums difficult and time consuming.

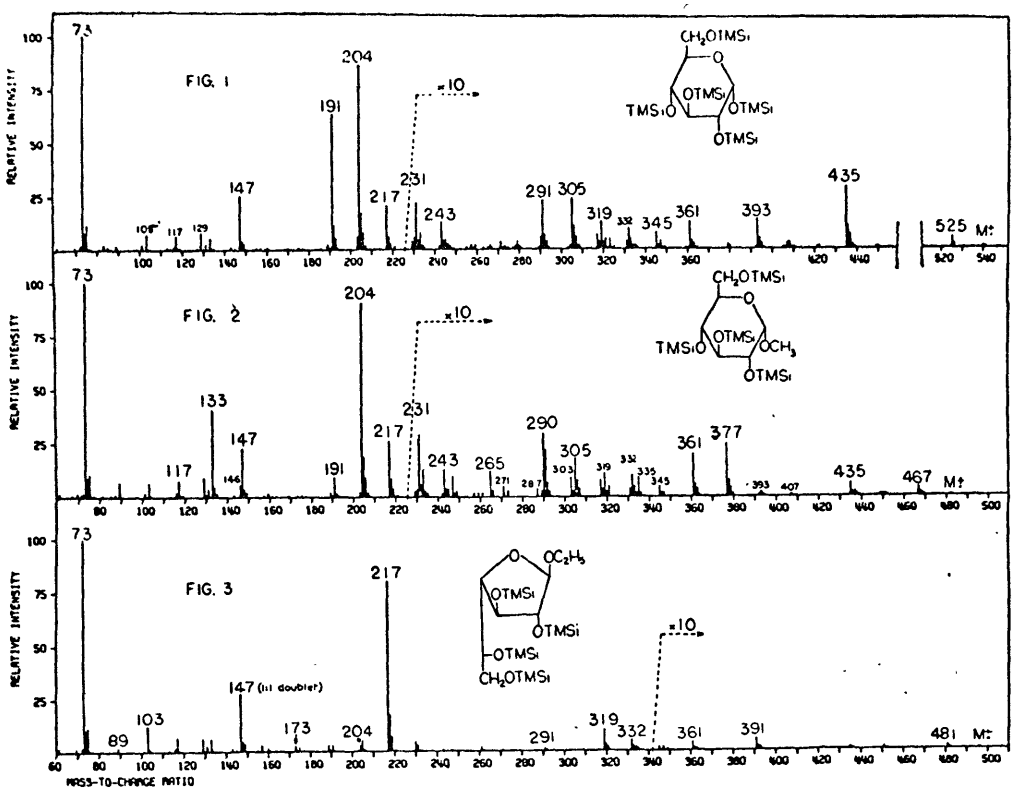
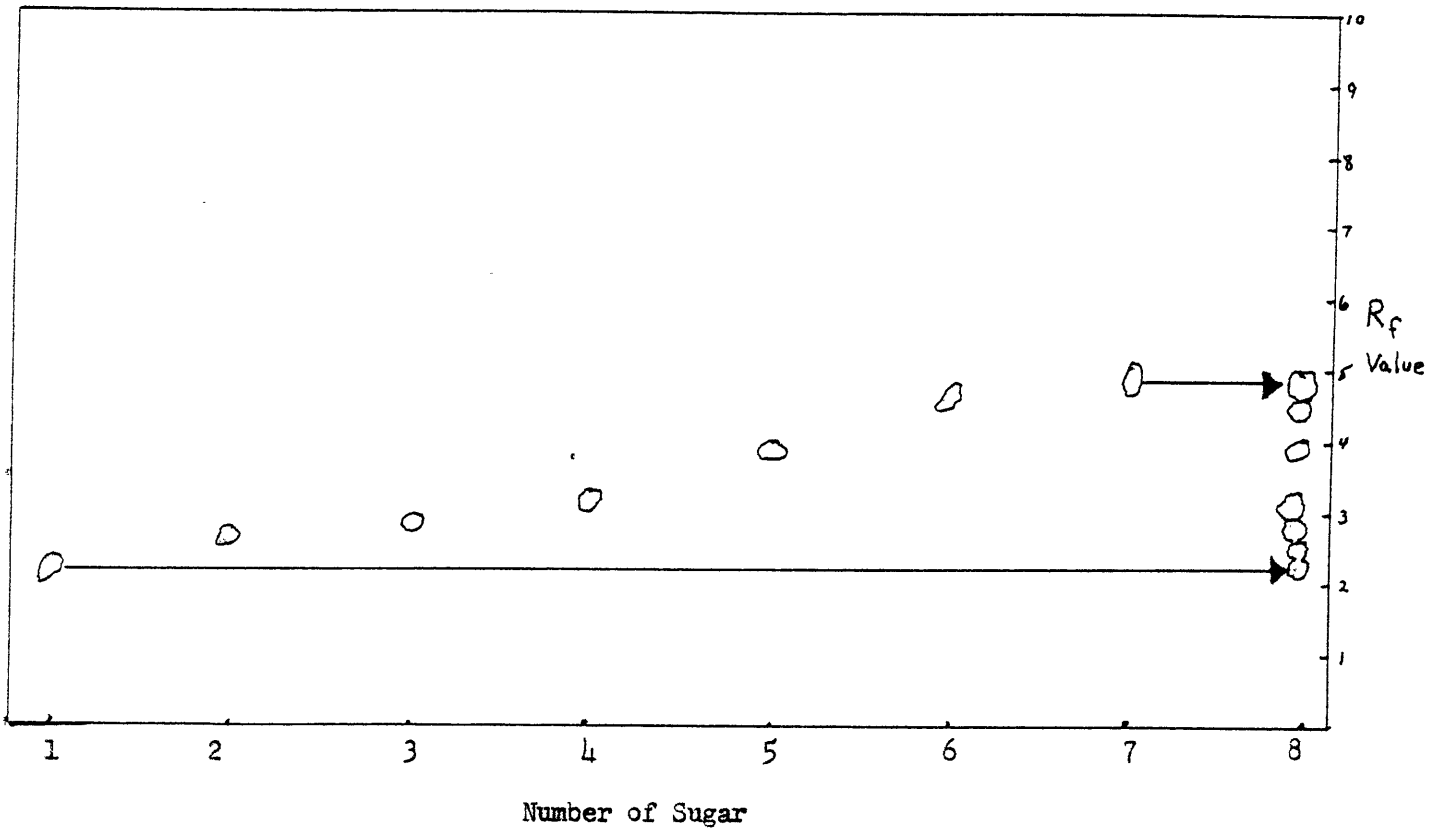


Figure 1. The 70-eV mass spectrum of 1,2,3,4,6-penta-*O*-trimethylsilyl-α-D-glucopyranose (1); molecular weight, 540.

Figure 2. The 70-eV mass spectrum of methyl 2,3,4,6-tetra-*O*-trimethylsilyl-α-D-glucopyranoside (12); molecular weight, 482.

Figure 3. The 70-eV mass spectrum of ethyl 2,3,5,6-tetra-*O*-trimethylsilyl-β-D-galactofuranoside (16); molecular weight, 496.

FIGURE 3. TLC separation of sugars.



Separation of monosaccharides on Eastman Chromogram Sheet 6061 (silica gel) impregnated with sodium acetate. Developed once with acetone/ chloroform/ methanol/ water (80:10:10:5); visualization: tetrazolium 'blue'. Samples: 1) galactose, 2) glucose, 3) mannose, fructose, 5) arabinose, 6) ribose, 7) xylose, and 8) mixture.

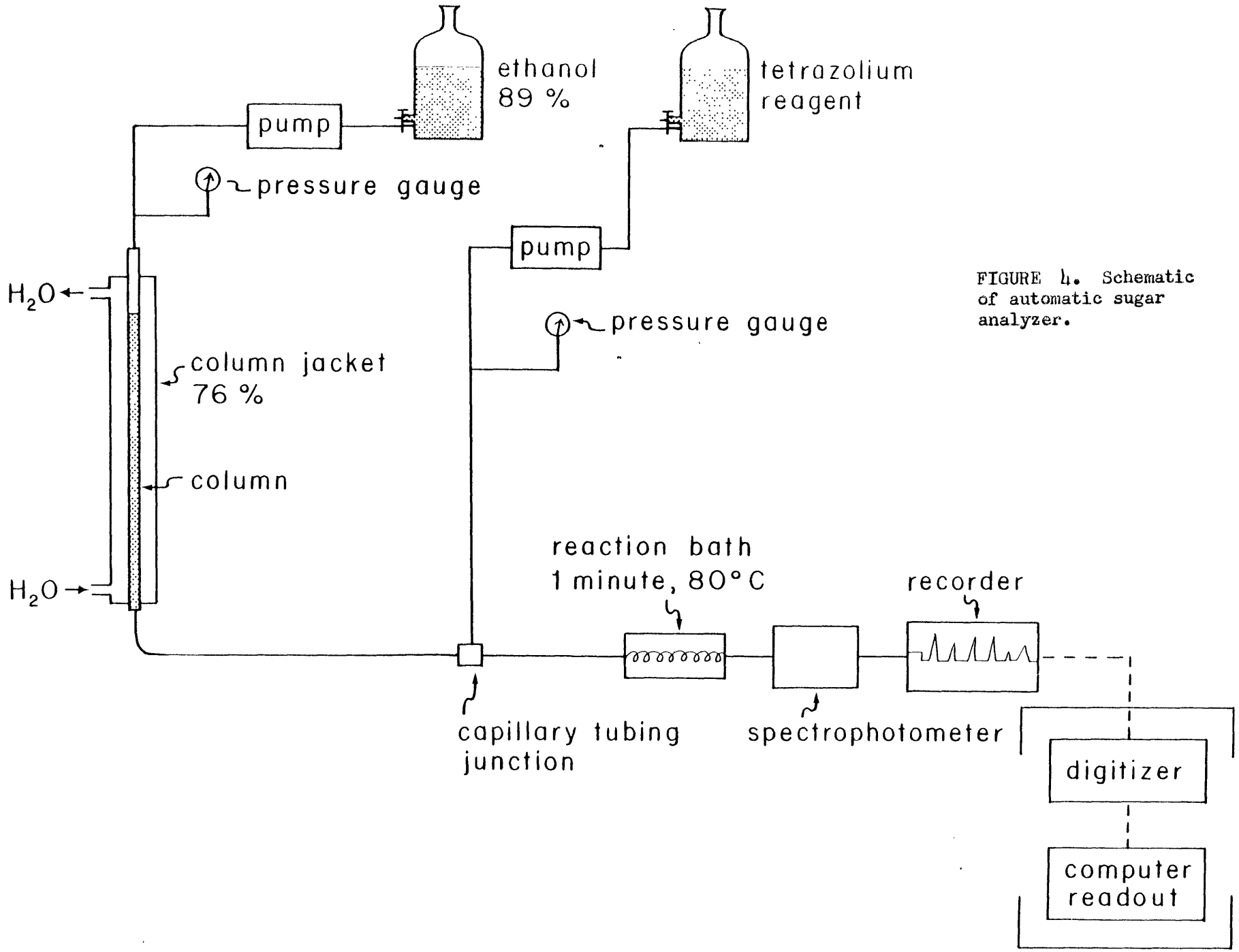


FIGURE 4. Schematic of automatic sugar analyzer.

FIGURE 5. Quantification of sugar analyzer by peak area. Slopes for most sugars are nearly identical which indicates that most sugars react on a mole for mole basis with the tetrazolium 'blue' reagent.

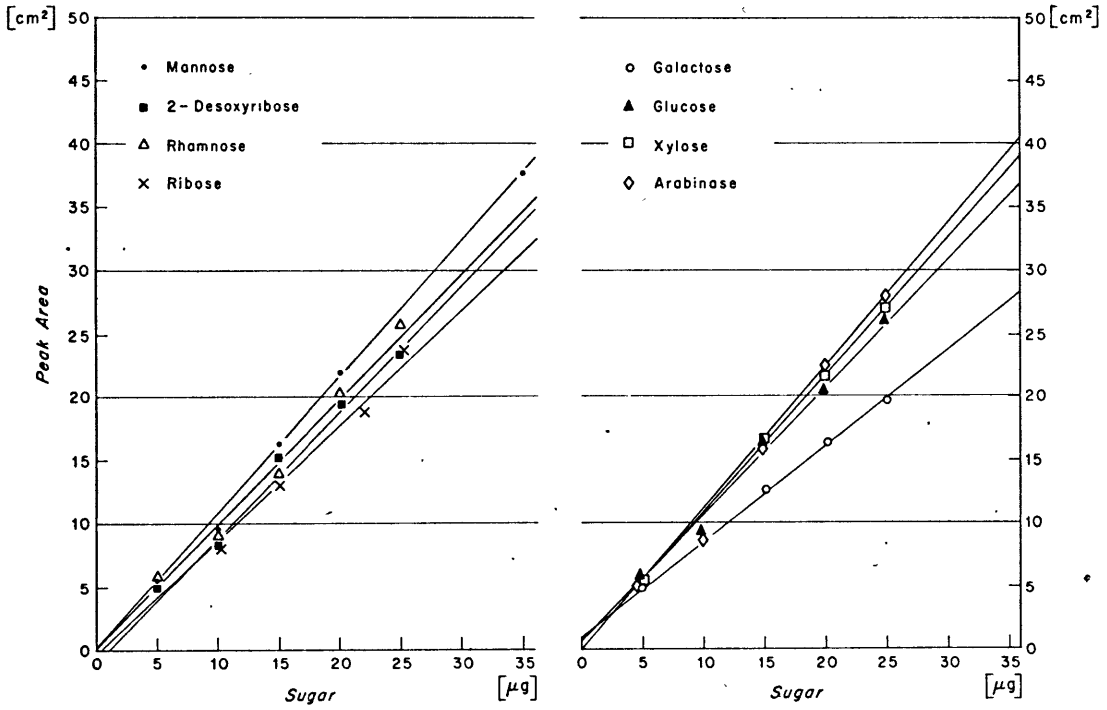


FIGURE 6. Influence of resin. Fine-grained, uniform resins gave the best resolution. 40 ug of desoxyribose (d-Ri), rhamnose (Rh), ribose (R), arabinose (A), xylose (X), manose (M), galactose (Ga), and glucose (Gl) were analyzed by:

A. Dowex 50W-X8 (37-74 u); resin bed 0.4 x 90 cm; column temperature 76° C; flow rate 0.5 ml/min; ethanol concentration 88%.

B. Technicon Type S (20 u); resin bed 0.28 x 110 cm; column temperature 76° C; flow rate 0.5 ml/min; ethanol concentration 88%.

FIGURE 6 A.

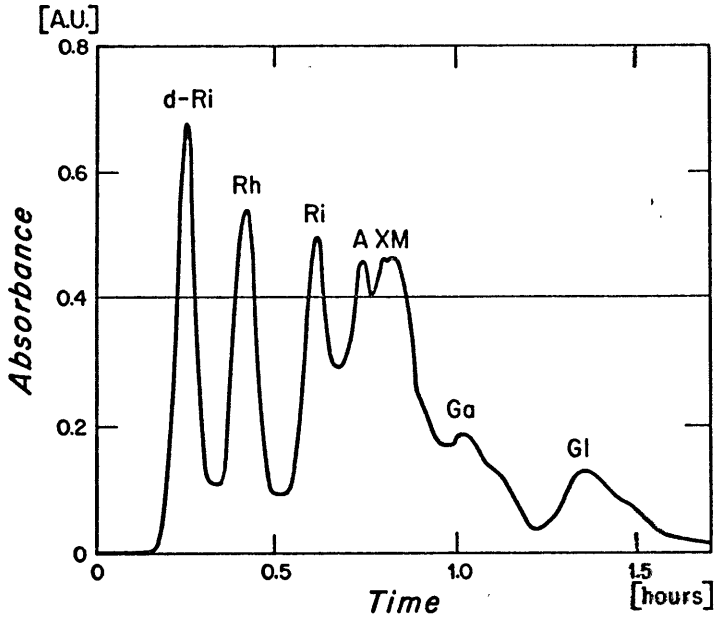


FIGURE 6B.

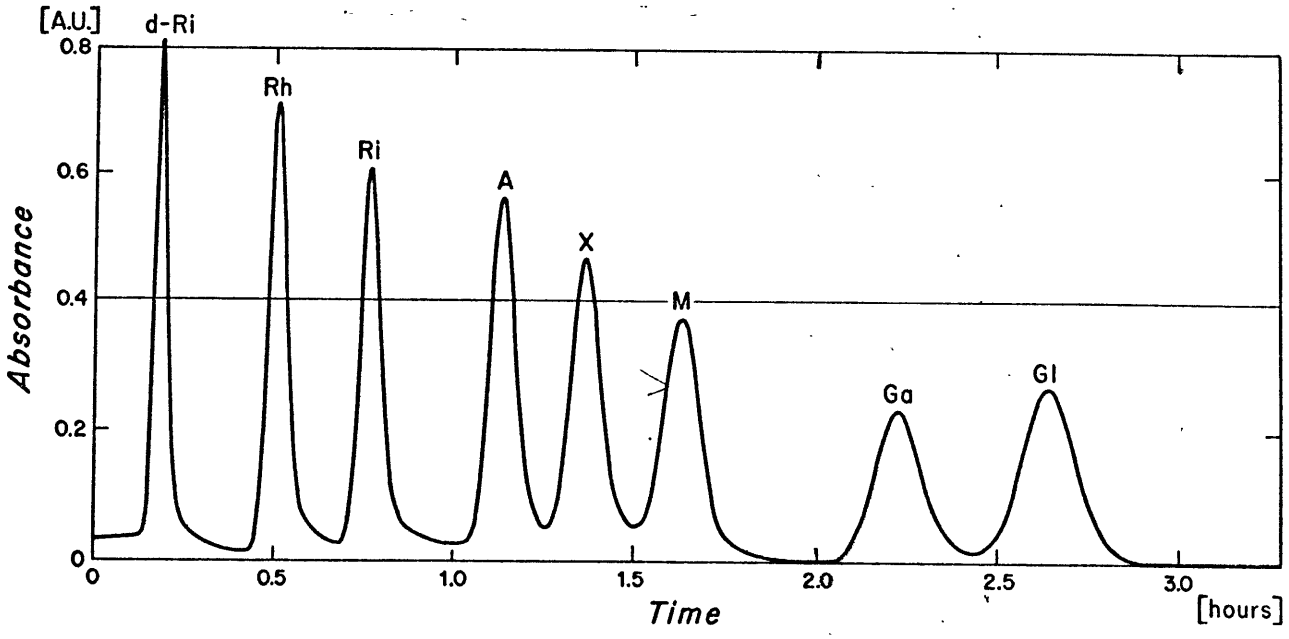


FIGURE 7. Influence of temperature. Peaks became sharper and better resolved at elevated temperatures.

A. 60° C; Technicon Type S resin; resin bed 0.28 x 110 cm; flow rate 0.5 ml/ min; ethanol concentration 88%; 20ug of each sugar.

B. 76° C; Technicon Type S resin; resin bed 0.28 x 110 cm; flow rate 0.5 ml/min; ethanol concentration 89%; 35 ug of each sugar.

Peak notation as in FIGURE 6.

FIGURE 7A. 60 ° C

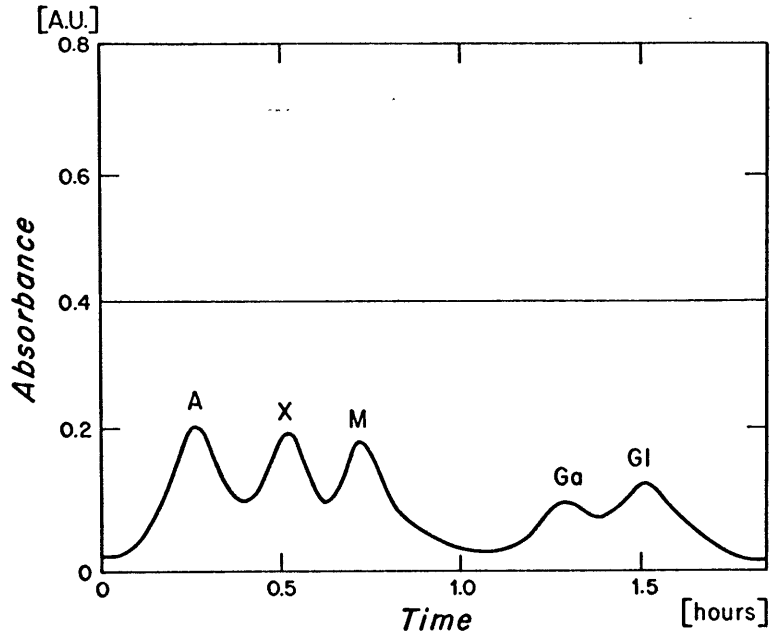


FIGURE 7B. 76 ° C

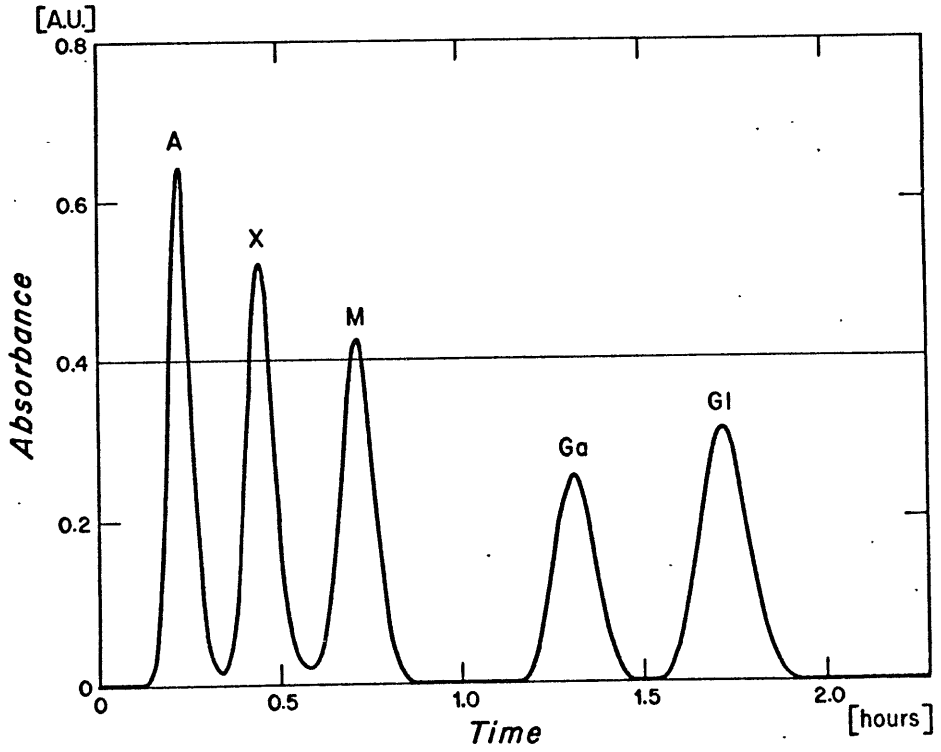


FIGURE 8. Influence of ethanol concentration of eluant. High ethanol concentrations led to increased retention times, large elution volumes, peak broadening, and excessive separations. The chromatogram depicted in this figure was run with 92% ethanol and was completed in three and one-half hours. Chromatogram B in FIGURE 7 (89% ethanol) was completed in two hours. Both analyses were run under identical conditions with the exception of the ethanol concentrations.

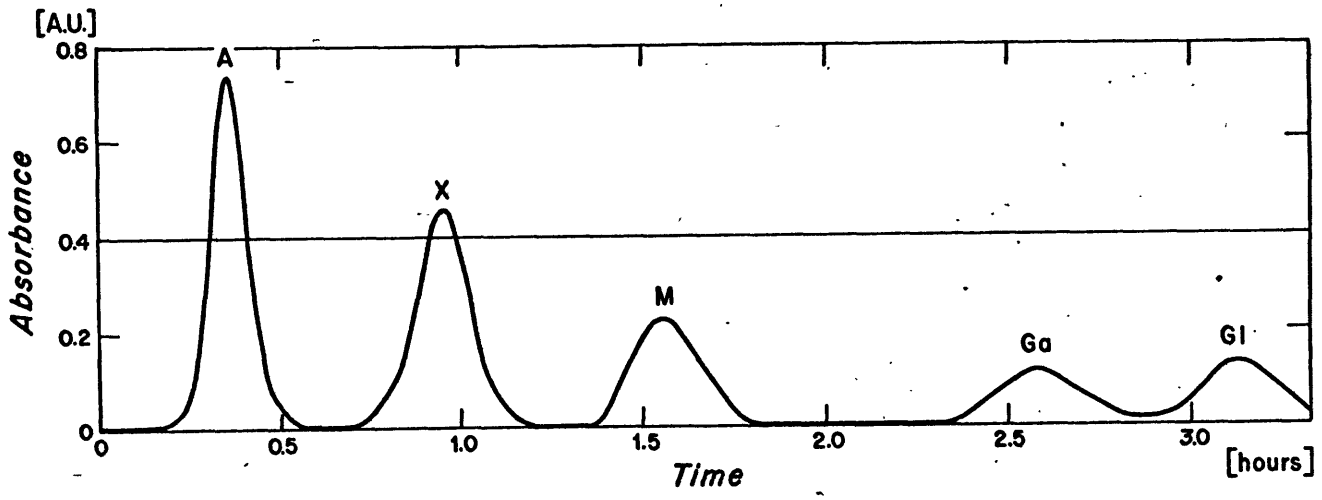


FIGURE 9. Influence of eluant flow rate. High flow rates led to decreased elution times and poor resolution. Xylose (x) and mannose (M) in chromatogram A are non-resolvable at a flow rate of 1.2 ml/min. However, these sugars are completely separated in chromatogram B which was run at 0.6 ml/min.

A. Flow rate = 1.2 ml/min; Technicon Type S resin; resin bed 0.28 x 110 cm; column temperature 76° C; ethanol concentration 88%; 20 ug of each sugar.

B. Flow rate = 0.6 ml/min; 40 ug of each sugar; the rest is the same as for FIGURE 9, A.

Peak notation as in FIGURE 6.

FIGURE 9A. Flow rate: 1.2 ml/min.

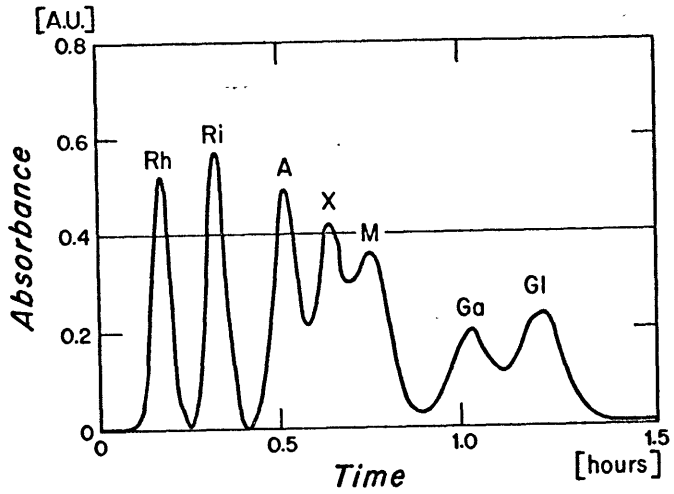


FIGURE 9B. Flow rate: 0.6 ml/min.

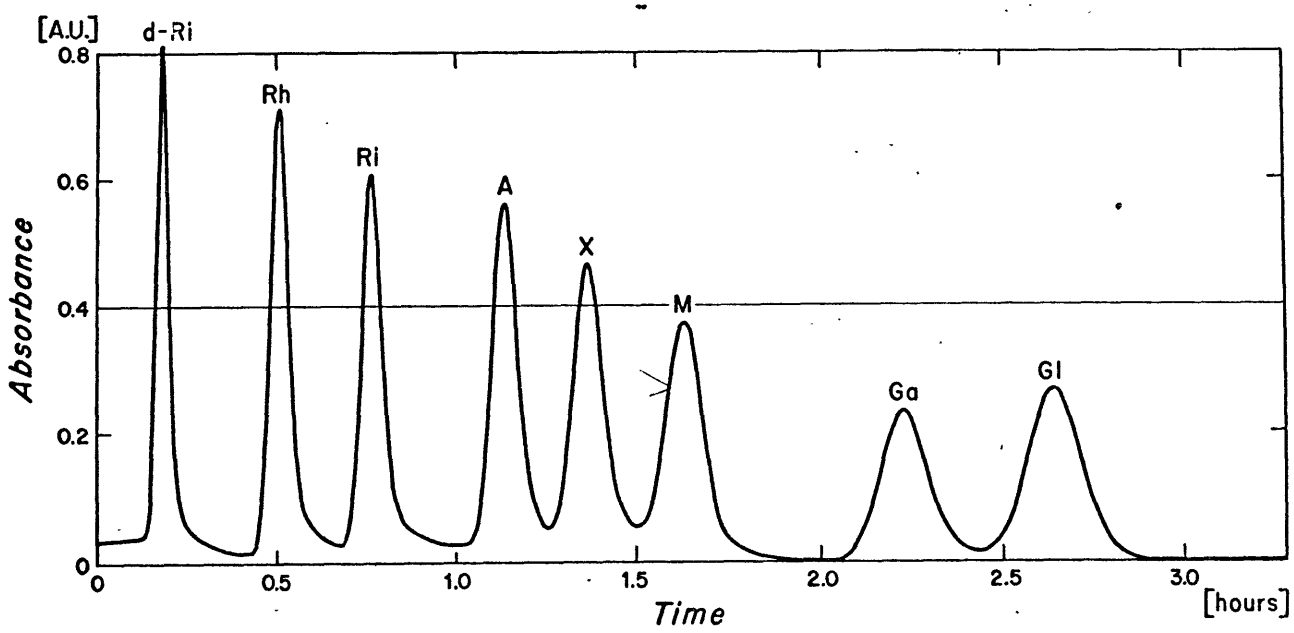


FIGURE 10. Analysis of a standard glucose solution with tetrazolium 'blue' in 60% ethanol. The absobances are linear for sugar concentrations under 50 ug/ml.

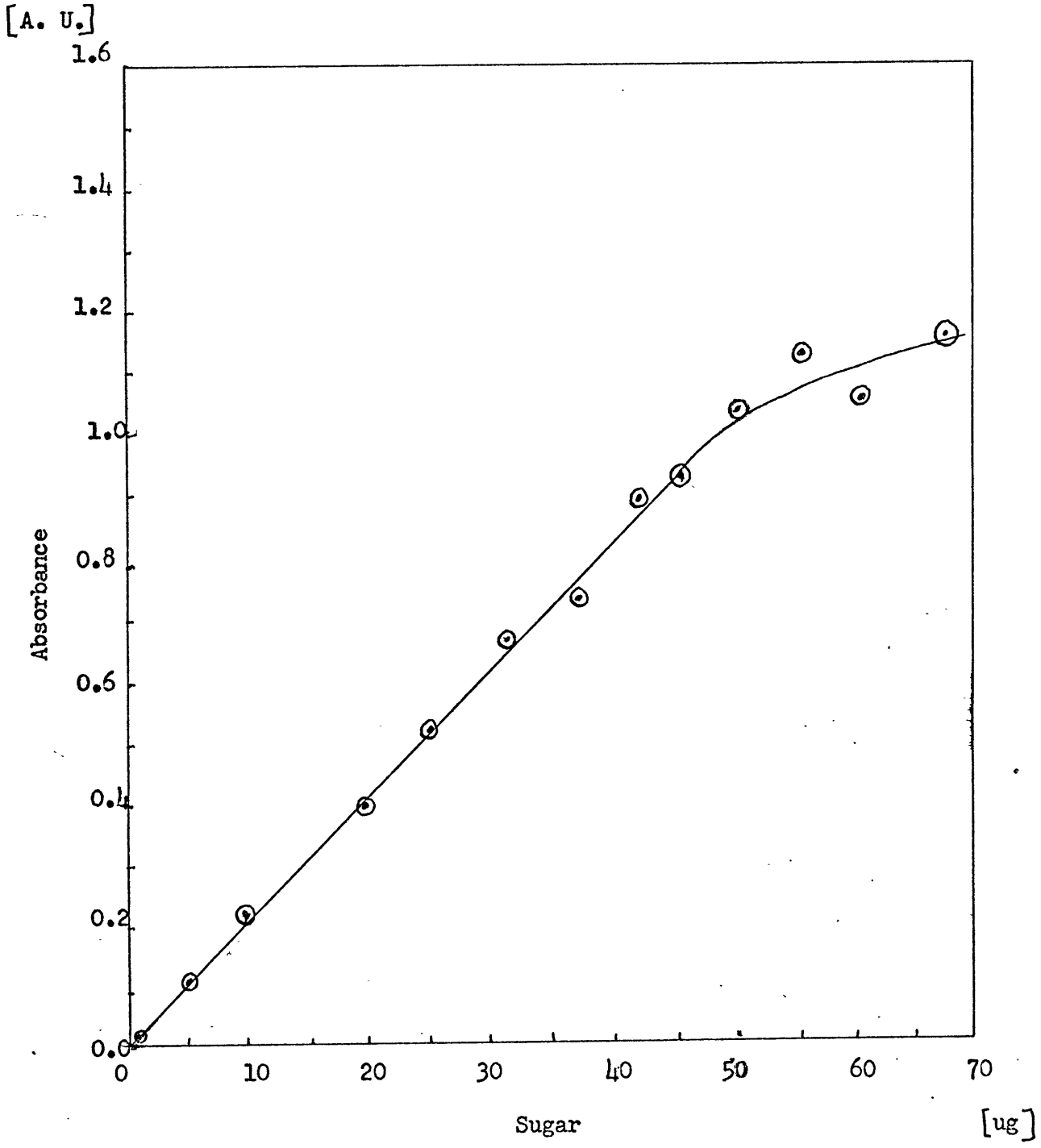


FIGURE 11. Absorbance versus wavelength of a standard sugar solution (20 ug/ml) dyed with tetrazolium 'blue.'

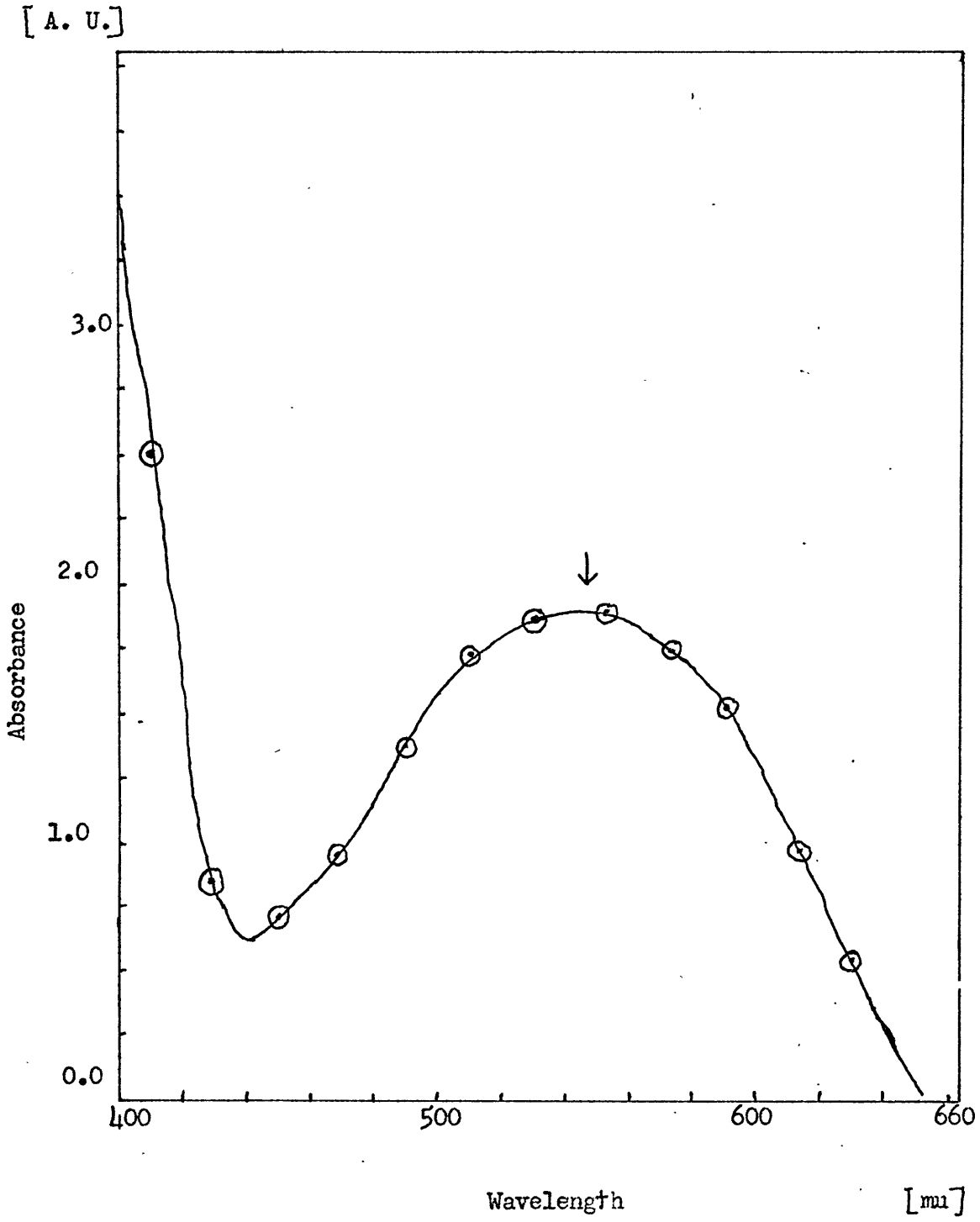


FIGURE 12. Automatic integration and digitization of peak areas recorded by a spectrophotometer. Numbers printed inside peaks were calculated automatically by by a Dual-Channel Digital Integrator (Infotronics, Inc.).

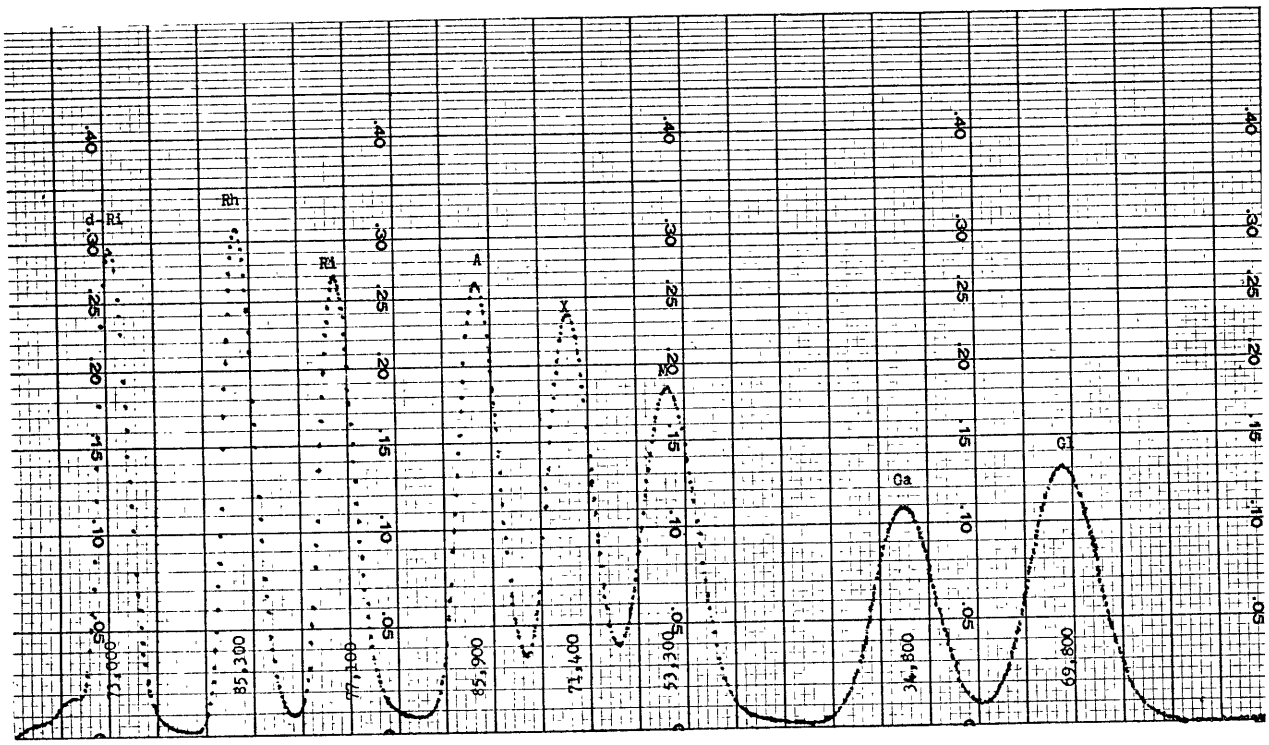


FIGURE 13 - Flow diagram for the extraction of soluble sugars from seawater.

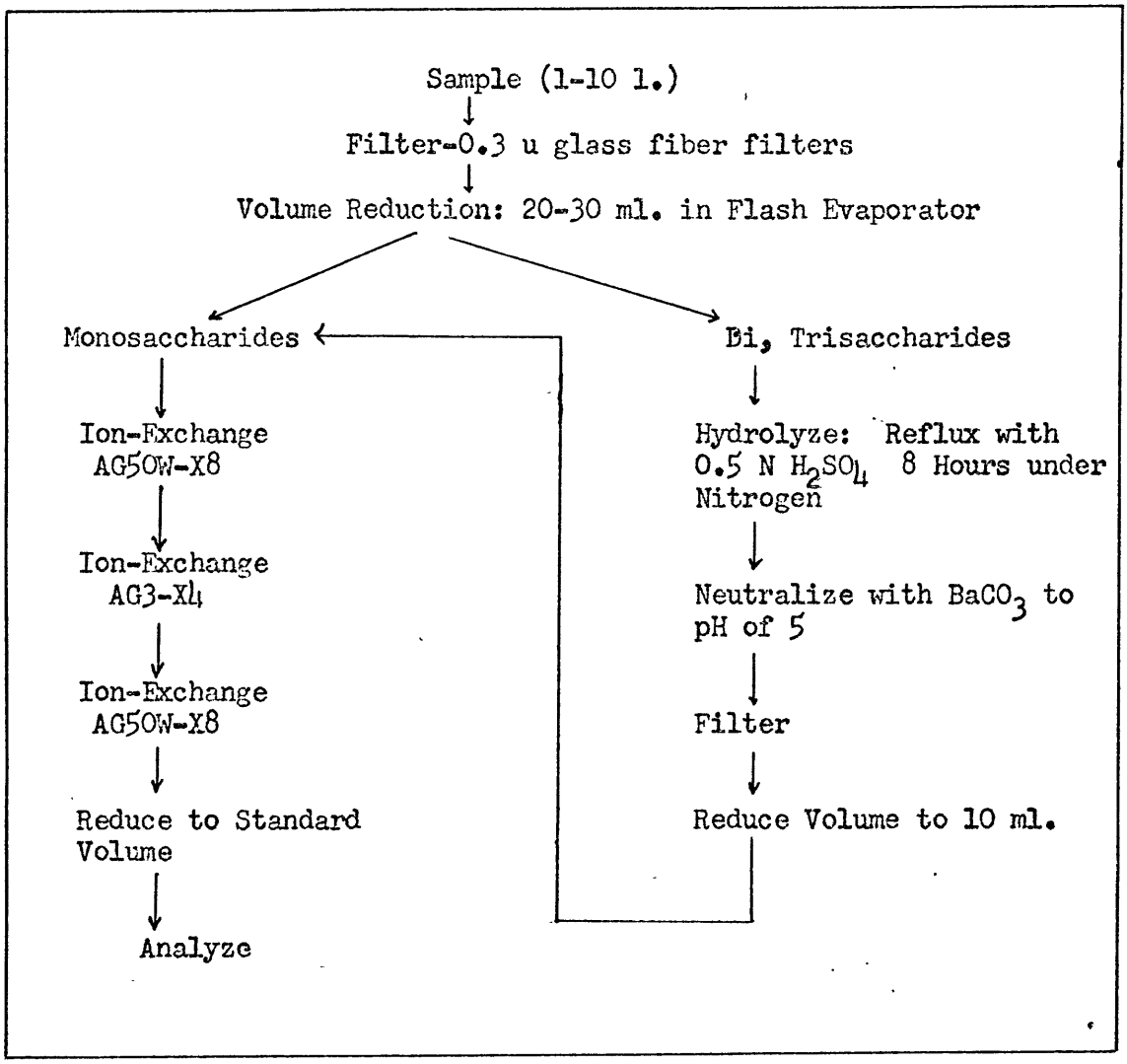


FIGURE 14. Analysis of a standard sugar solution, 20 ug. of each: desoxyribose(d-Ri), rhamnose (Rh), ribose (Ri), arabinose (A), xylose (X), mannose (M), galatose (Ga), glucose (Gl). Running conditions in text.

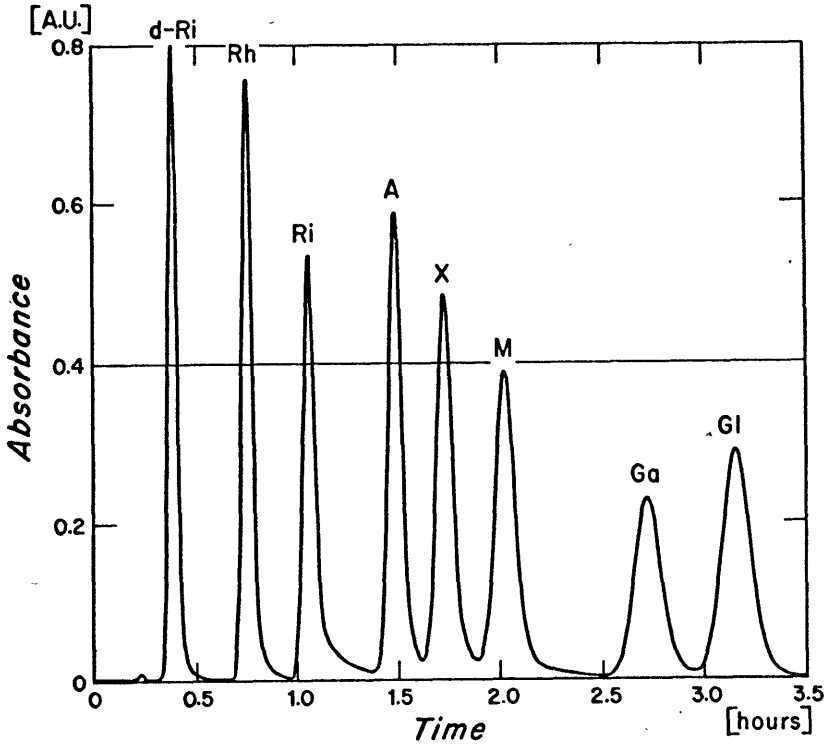


FIGURE 15. Test no.1 of desalting resins. An aliquot of the standard depicted in FIGURE 14 was eluted through the desalting columns with 300 ml of H_2O. Desoxyribose was 80% destroyed, and ribose, 30%.

FIGURE 16. Test no.2 of desalting resins. Desoxyribose and ribose were eluted through the desalting columns with 300 ml of H_2O. In addition to the loss of these sugars, new sugars (rhamnose, arabinose, and xylose) were produced in trace quantities.

FIGURE 15. Test no. 1 of desalting resins.

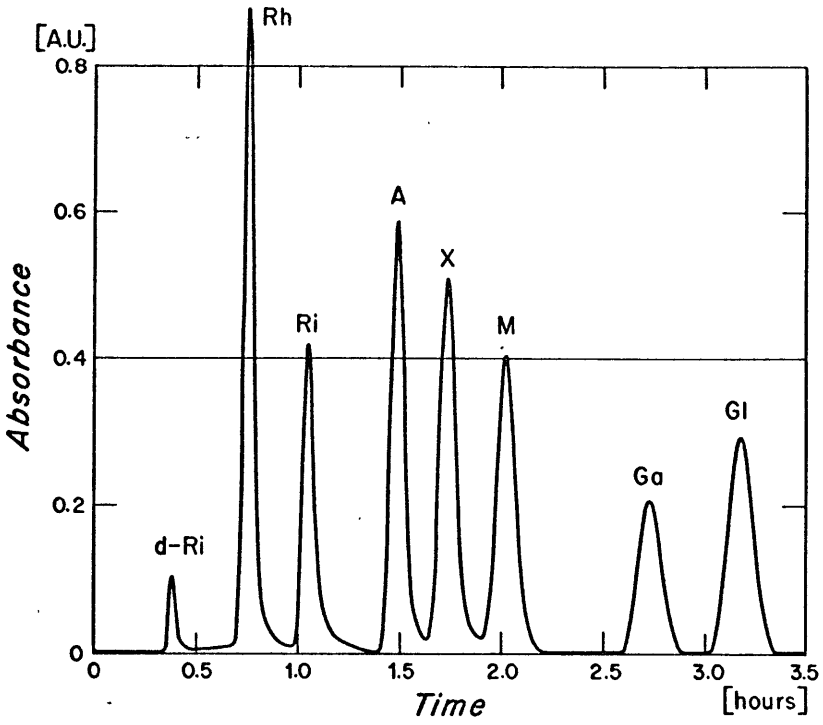


FIGURE 16. Test no. 2 of desalting resins.

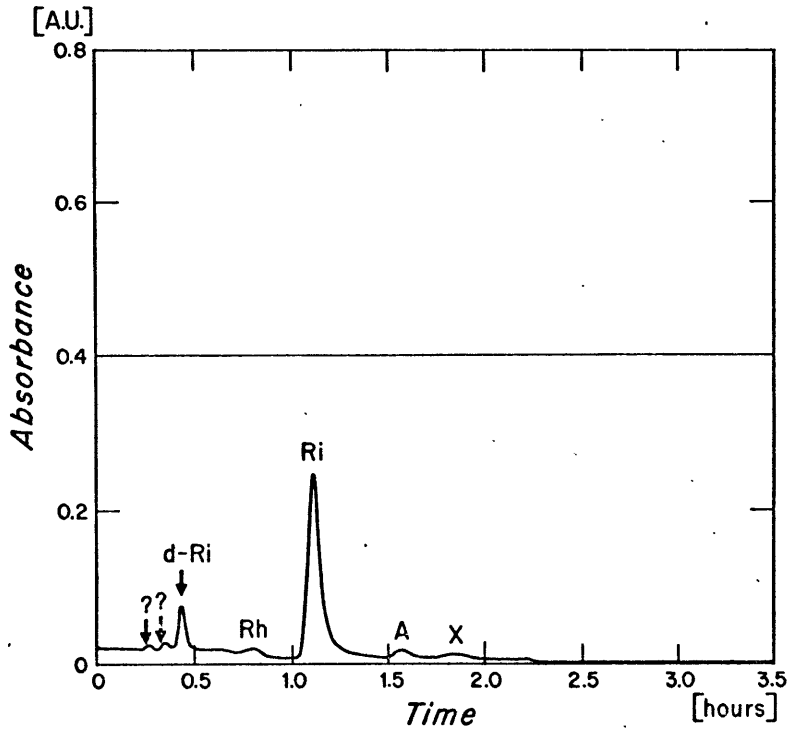
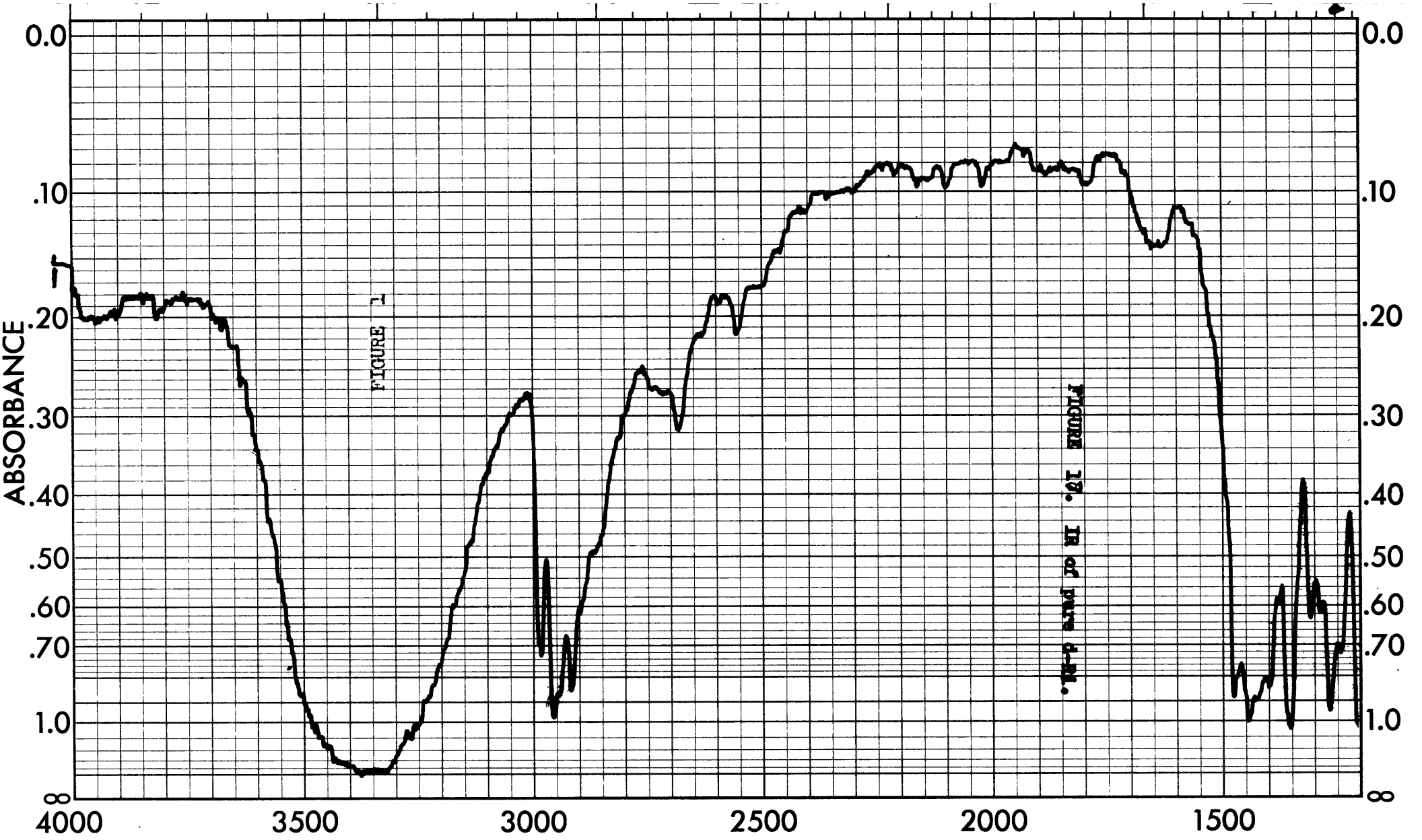


FIGURE 17. IR spectrum of pure desoxyribose. This spectrum resembles that of the other monosaccharides except for the absorbance at 1450 cm^{-1} which is due to methylene bending.

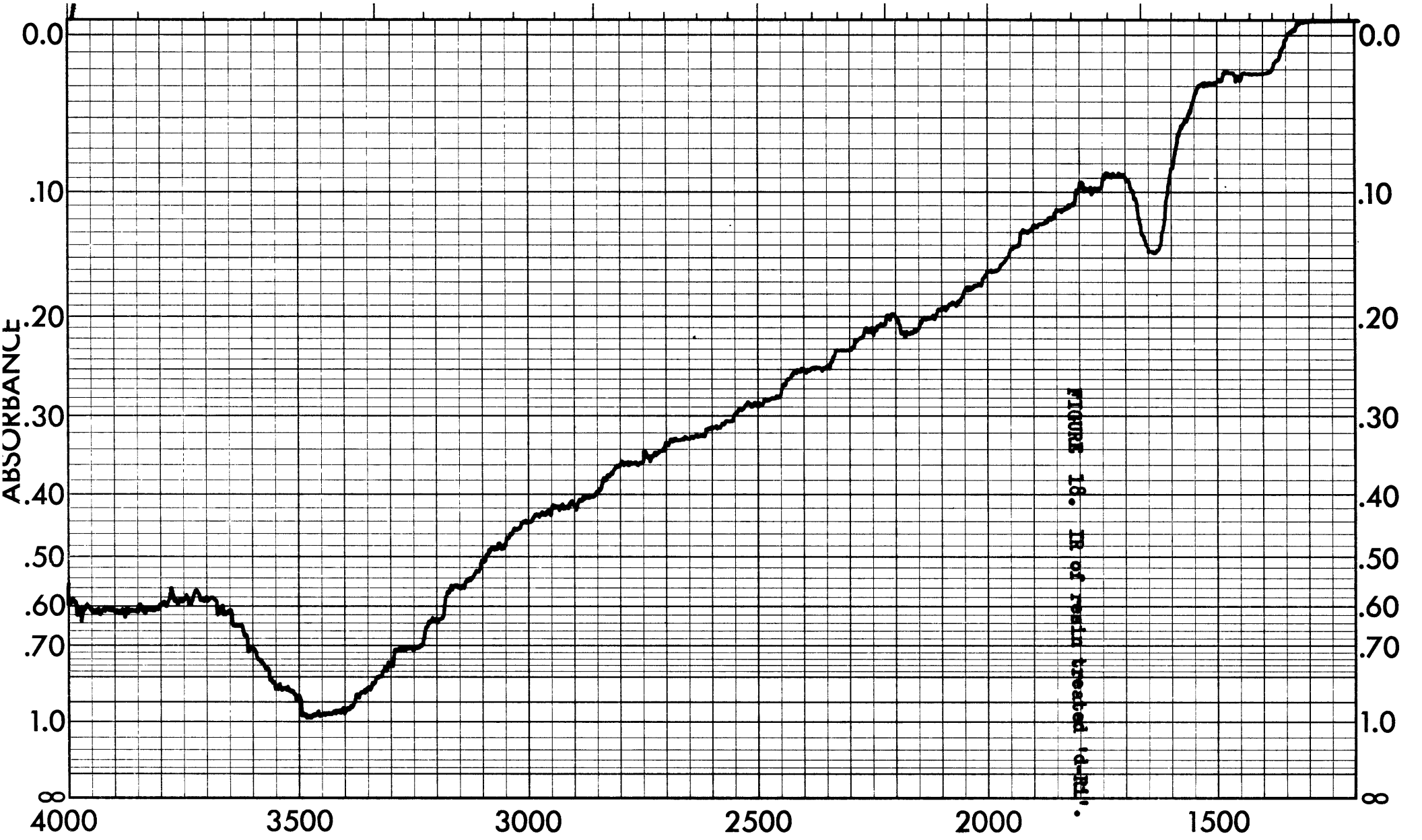
FIGURE 18. IR spectrum of 'desoxyribose' passed through desalting columns. By comparison with FIGURE 17 complete destruction (fragmentation) of desoxyribose is evident.



SAMPLE 2-Deoxyribose	CURVE NO. 1	SCAN SPEED	OPERATOR GRH
	CONC.	SLIT	DATE 6/26/20
ORIGIN	CELL PATH	REMARKS	
SOLVENT KBr Pellet	REFERENCE air		

PART NO. 337-1207

PERKIN-ELMER ®



SAMPLE Deoxyribose passed thru ion exchange	CURVE NO. 2	SCAN SPEED	OPERATOR GRH
	CONC.	SLIT	DATE 6/26/70
ORIGIN	CELL PATH	REMARKS	
SOLVENT KBr Pellet	REFERENCE air		

FIGURE 19 - Flow diagram for the extraction of soluble sugars from sediment.

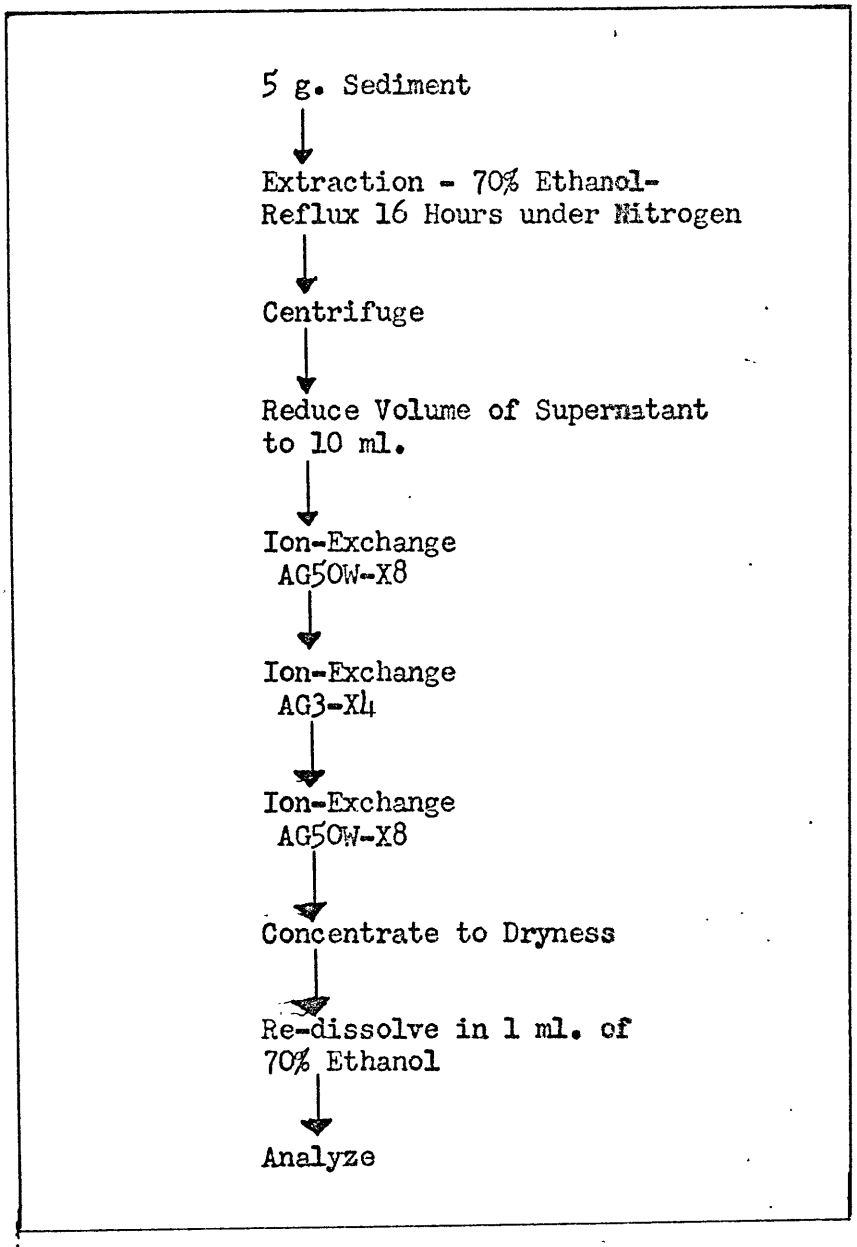


FIGURE 20. H_2O extraction of a segment of Black Sea core 1474K (46-47 cm.)

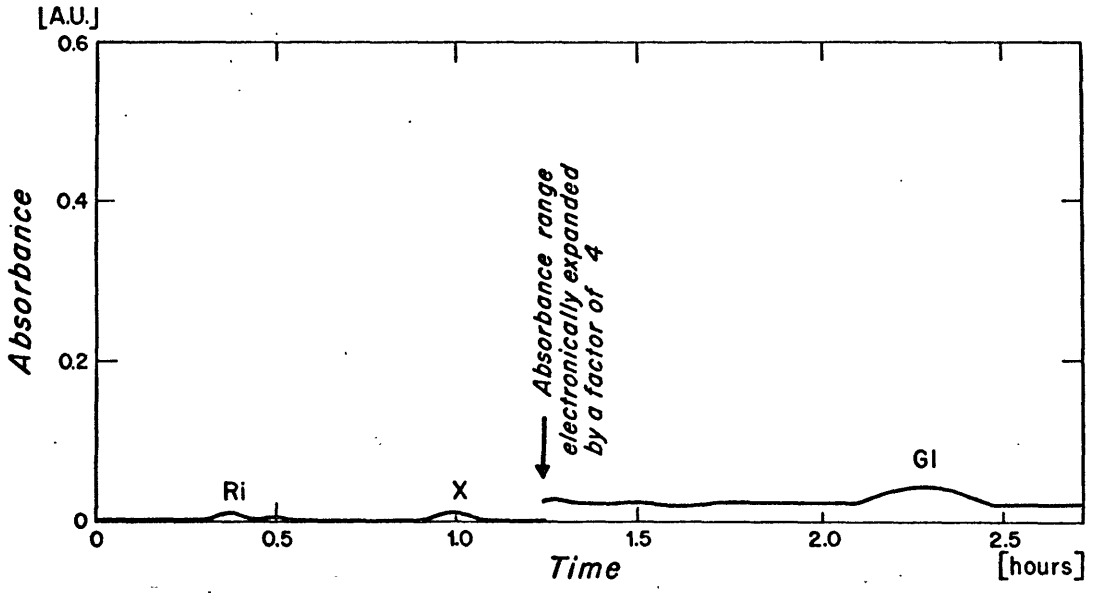


FIGURE 21. 70% ethanol extraction of a segment of Black Sea core 1474K (46-47 cm.). By comparing this figure with the chromatogram of the H_2O extraction (Figure 20), it is evident that the ethanol extraction was considerably more efficient.
Pentoses predominate over hexoses. In addition, fucose (Fu) is characteristic only of certain marine algae (Whistler and Smart, 1953).

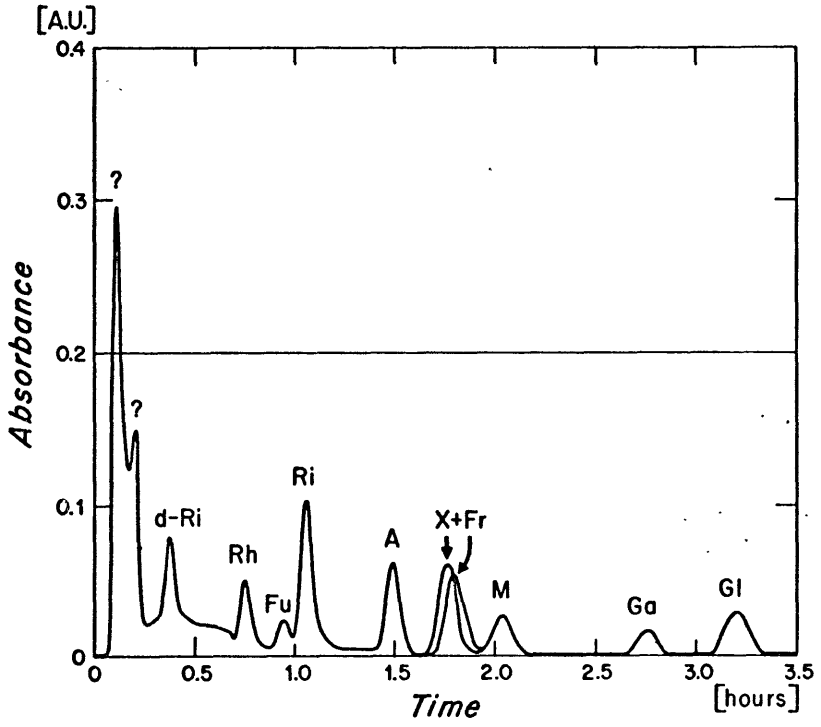
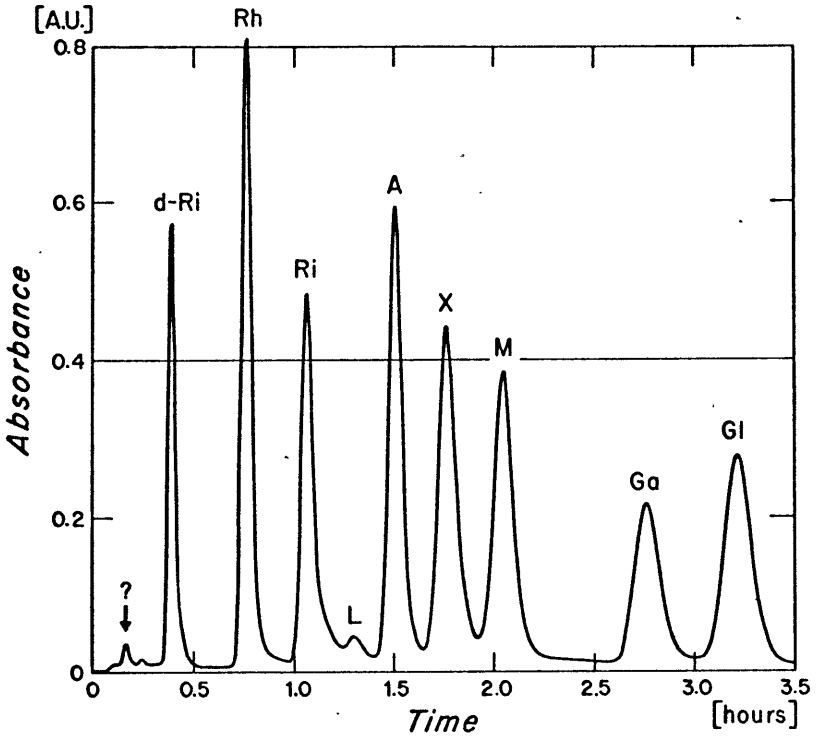


FIGURE 22. Test of the effects of ethanol extractions on a standard sugar solution. A slurry of ignited sediment (800° C for 3 hours) and an aliquot of a standard solution (depicted in Figure 14) were refluxed for 16 hours with 70% ethanol. Minor destruction of the sugars occurred, however, lyxose and one as yet unidentified sugar were produced.



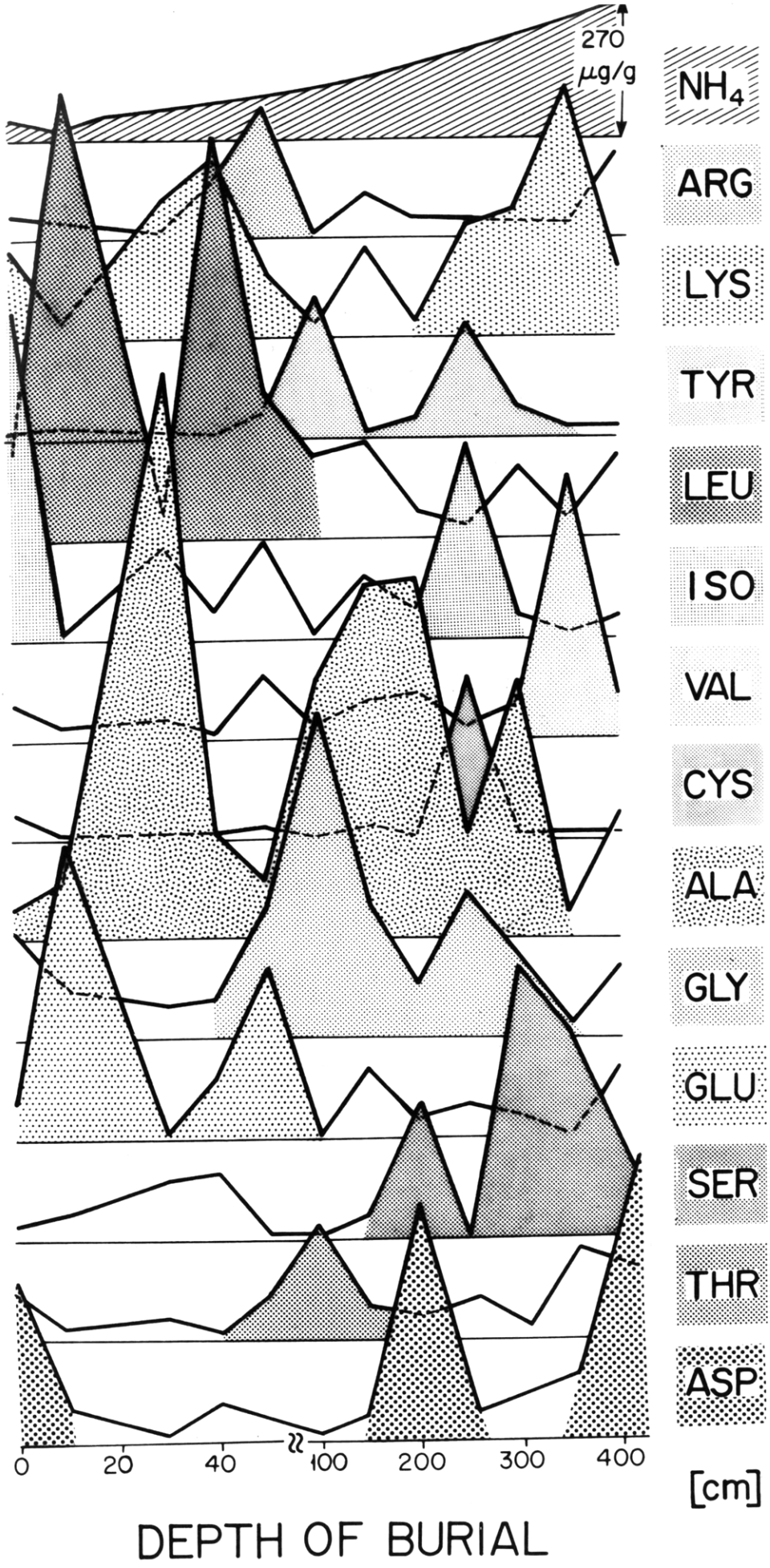


FIGURE 23. Separation of amino acids along a vertical profile of Santa Barbara sediments off the coast of California (Degens, 1967).

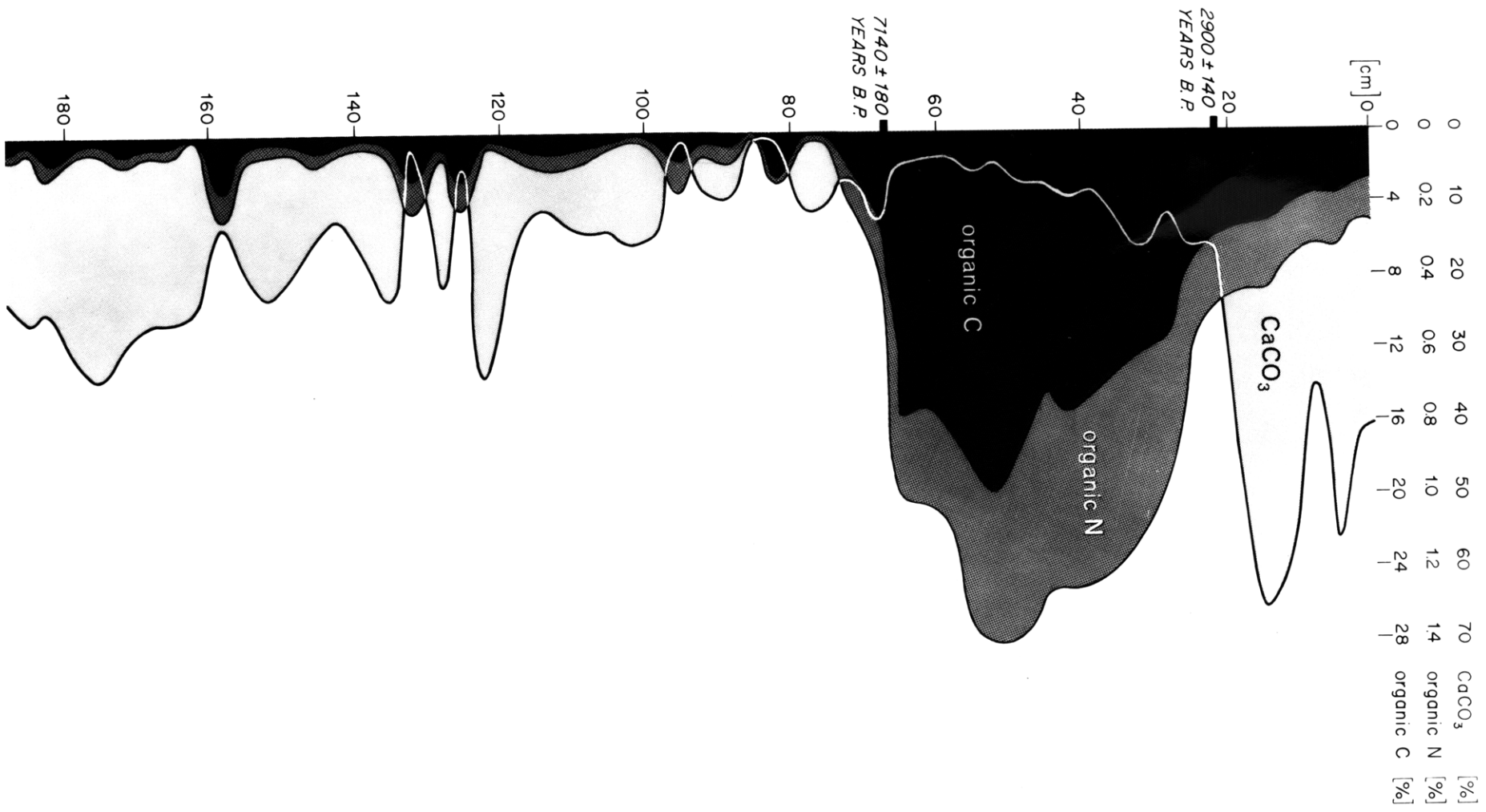


FIGURE 24. Percent organic carbon and nitrogen, and $CaCO_3$ in Black Sea core 1474K. The segment between 30 and 70 cm. is being analyzed in detail in hopes of detecting chromatographic separation of free sugars (Figure 23).

FIGURE 25. Test no.1 of acid hydrolysis procedures. An aliquot of a standard sugar solution (depicted in FIGURE 14) was refluxed in 1 N H_2SO_4 for 8 hours under a blanket of nitrogen. Only desoxyribose was appreciably affected.

FIGURE 26. Test no.2 of acid hydrolysis procedures. An aliquot of a standard sugar solution (depicted in FIGURE 14) plus two grams of ignited sediment (800° C for 3 hours) were refluxed for 8 hours in 1 N H_2SO_4. All sugars were affected to varying degrees as a result of catalytic destruction on the mineral (clay) surfaces.

FIGURE 25. Hydrolysis test: 1N H_2SO_4.

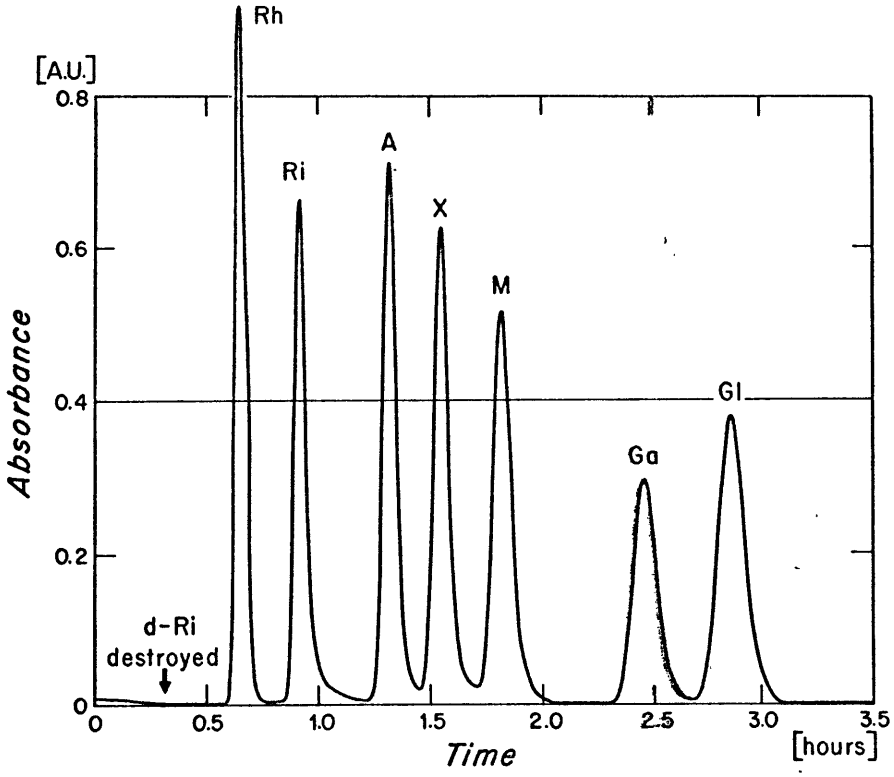


FIGURE 26. Hydrolysis test: 1N H_2SO_4 plus 2 g. ignited sediment.

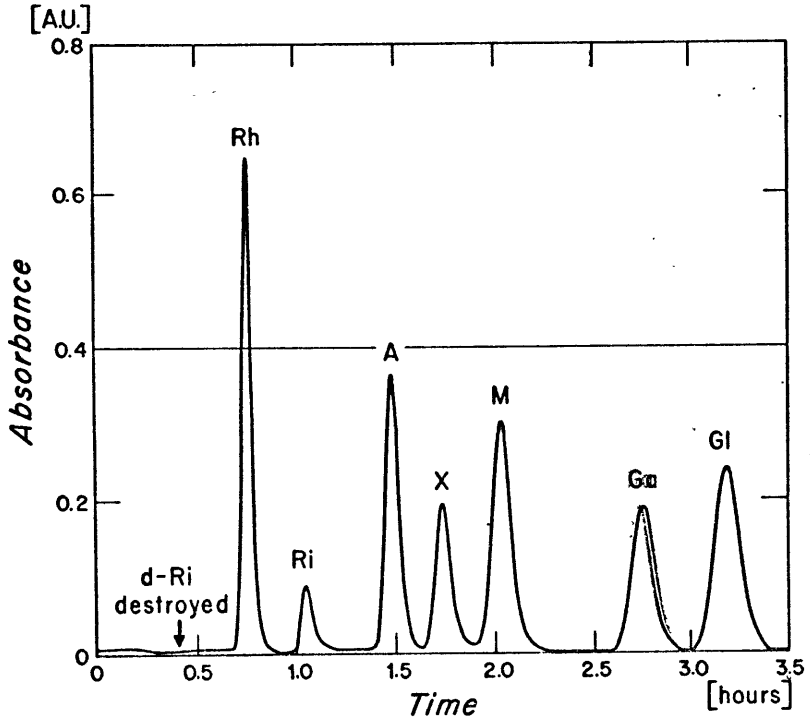


FIGURE 27. Test no.3 of acid hydrolysis procedures. An aliquot of a standard sugar solution (depicted in FIGURE 14) was refluxed for 8 hours in 6 N HCl. All sugars were > 95% destroyed.

FIGURE 28. Test no.4 of acid hydrolysis procedures. An aliquot of a standard sugar solution (depicted in FIGURE 14) was refluxed for 8 hours in 1.5 N HCl. This analysis indicates that sugars are sensitive to dilute HCl.

FIGURE 27. Hydrolysis test: 6N HCl.

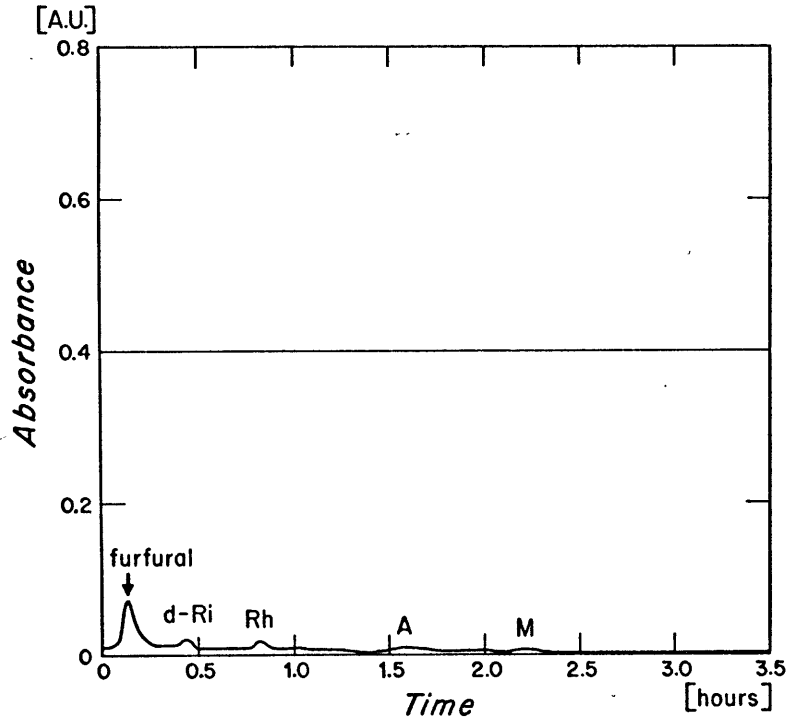


FIGURE 28. Hydrolysis test: 1.5N HCl.

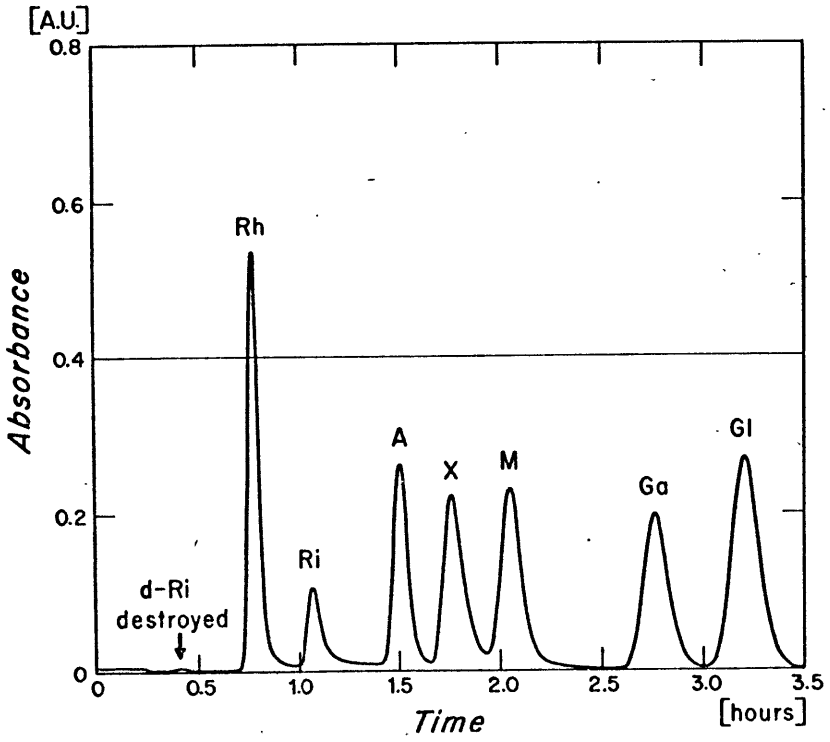


Table 1 Retention Times

quantity of sugar (ug)	retention time:distance of peaks from d-Ri in cm.							
	d-Ri	Rh	Ri	A	X	M	Ga	Gl
5	0	4.9	8.7	14.3	17.9	21.8	31.1	37.4
10	0	4.7	8.5	13.9	17.4	21.2	-	36.3
15	0	4.8	8.6	14.0	17.4	21.3	30.3	36.5
20	0	4.8	8.9	14.5	18.1	22.3	31.7	38.2
25	0	5.0	8.9	14.5	18.1	22.1	31.3	37.7

Table 2 Sugar Losses on Desalting Columns

eluant	percent loss							
	d-Ri	Rh	Ri	A	X	M	Ga	Gl
H_2O	80	0	20	10	0	0	0	0
70% ethanol	50	0	20	20	0	0	0	0

Table 3. Losses of Sugars during Acid Hydrolysis Tests.

acid	fractional loss							
	d-Ri	Rh	Ri	A	X	M	Ga	Gl
1N H_2SO_4	1.0	0	0	0	0	0	0	0
1N H_2SO_4 plus 2 g. ignited sediment	1.0	.4	.8	.7	.7	.6	.3	.3
6N HCl	.95	.95	1.0	.95	1.0	.95	1.0	1.0
1.5N HCl	1.0	.2	.8	.5	.6	.3	.2	.2