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Doctor's Dissertation

A Study of Some Reaction Rates in the
Homogeneous System Water-Sodium
Hydroxide-Cellobiose

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A STUDY OF SOME REACTION RATES IN THE HOMOGENEOUS
SYSTEM WATER-SODIUM HYDROXIDE-CELLOBIOSE

A thesis submitted by

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PREFACE

This doctoral thesis and the related academic program had its Genesis some forty years ago. An interest in the industrial potential of the great forests of British Columbia was shaped and sharpened to a desire to be a part of the pulp and paper industry as a Chemical Engineer. During undergraduate days, the shaping included a summer in the bush on a timber cruising party and summers (and a winter because of the great depression of 1929 and onwards) in the Woodfibre and Port Alice mills of the then British Columbia Pulp and Paper Company, Ltd. At Woodfibre, the Mill Manager, the late E. P. Brennan, strongly encouraged and in everyway helped the shaping of the course and permanently catalyzed continuance on it.

The forty years have now passed. They ran a pattern of advances, retreats, wars, mergers, alliances, begats, and wanderings. Having now returned to terra academica, it will perhaps be excusable to refer in an analogous sense to the thesis presented in the following pages as the Revelations.

Many have helped all along the pathway of this study. The continuing interest and support of all the Staff and Faculty of The Institute of Paper Chemistry was outstanding. Dean Lewis and then Dean Whitney could always find ways of accommodating a somewhat itinerant student. Dr. Louis Wise, in particular, and many other members of the faculty, and the Candidates Committee, Drs. John Green, Bert Browning, Norman Thompson, and Harry Wilder never failed to be interested and to find time to discuss, evaluate, and encourage.

Financial support from The Institute of Paper Chemistry, the University of Victoria, and Sandwell and Company, Ltd. was an essential part of the total operation and is gratefully acknowledged.

Above and beyond was the unfailing and understanding support of my wife, Julia, without which the program could not have been carried out. She effectively took the

helm at home for the two years I was away in academic residence. She always shared with me a steadfast resolve to press on.

To Julia and all who shared and helped, my very grateful thanks.

SUMMARY

It was established that the transformation and degradation of cellobiose (7) at 0.001M in 1M NaOH at 22°C. under N₂ in the dark gave rise to D-glucose (1), D-fructose (2), D-mannose (3), cellobiulose (8), and 4-O-D-glucosylmannose (9). No other neutral carbohydrates in amounts greater than 0.5% of the starting material were found.

Rates of the isomerization, epimerization, and degradation reactions occurring under the above conditions were measured in systems using each of these six carbohydrates in turn separately as starting material. For these kinetic studies, cellobiulose (4-O-D-glucosylfructose) was prepared by a modified procedure. Simplifying modifications to a method for the preparation of 4-O-D-glucosylmannose were introduced and an improved yield was obtained.

The materials of interest in reaction systems were resolved by column chromatography using anion resins in the borate form. Separation and elution of the carbohydrates required about nine hours using a graded eluant. Elution began with 0.12M H₃BO₃ at pH 8.50 by adjustment with KOH and ended with 0.15M K₂B₄O₇. Mannose-fructose and glucosylmannose-glucosylfructose, were difficult to separate. Separation of these four required a very specific elution gradient. The column effluent was monitored continuously and analyzed colorimetrically using automated Technicon equipment. Absorbance was measured at 410 nm. using orcinol/sulfuric acid as the color forming reagent. The chromatography-colorimetric analysis sequence had a precision of about $\pm 0.5\%$. The accuracy of single determinations of the weight of a carbohydrate was about $\pm 2\%$.

Examination of the experimental data showed that for each starting material its rate of disappearance was pseudo-first-order during the initial stages of the reaction. Reaction rate constants were then abstracted from the appropriate reaction

rate equations by a stepwise sequence. The first step was to obtain approximations of the reaction rate constants for the glucose-fructose-mannose monomer system by algebraic manipulation of the necessary rate equations and consideration of initial rates. These approximations were then refined by a reiterative mechanical process using an IBM 1620 computer in conjunction with a Calcomp 565 Digital Incremental Plotter. The values of the rate constants as parameters in an analog-simulator program (PACTOLUS) were slowly adjusted until the computer plotted concentration vs. time curves were a visual best fit with the corresponding experimental data.

This same sequence of approximation and refinement was repeated for the cellobiose-cellobiulose-4-O-D-glucosylmannose disaccharide system. Then the rate constants for the overall disaccharide-monomer system were given a final refinement on the computer. The computer-plotted concentration vs. time curves from these rate constants were a good fit with the experimental data. The final values (hr.^{-1}) of the reaction rate constants obtained by this stepwise sequence were: k_{12} 0.036, k_{13} 0.0005, k_{15} 0.002, k_{21} 0.038, k_{23} 0.006, k_{24} 0.072, k_{31} 0.0005, k_{32} 0.011, k_{36} 0.002, k_{78} 0.078, k_{79} 0.0005, $k_{7,10}$ 0.002, k_{87} 0.022, k_{89} 0.003, k_{81} 0.065, $k_{8,12}$ 0.023, k_{97} 0.002, k_{98} 0.013, and $k_{9,11}$ 0.006, where (4), (5), (6), (10), (11), (12) are products other than (1), (2), (3), (7), (8), (9) from each of these carbohydrates, respectively.

It was found that under the conditions used the values of the rate constants of all the reversible transformation reactions occurring in the disaccharide system were essentially the same as the counterpart reactions in the monomer system with the one exception that the rate constant for the cellobiose to cellobiulose isomerization was about double that of the glucose to fructose isomerization. Presumably, the glucosyl entity on the C4 of the biose had a significant effect in this reaction. It was also found that all the values for these

isomerization reactions were significantly larger than values for epimerization reactions, and all pathways to and from mannose or glucosylmannose were relatively unfavorable. The "peeling" reaction occurred as expected though only about 80% of the consumed cellobiose molecules were peeled.

THE PROBLEM

The broad objective of the study was to gain further knowledge of the reaction rates and mechanisms by which carbohydrates, particularly $\beta(1\rightarrow4)$ glucans, are transformed and degraded in aqueous alkaline solutions.

While the isomerization, epimerization, and degradation of carbohydrates has been extensively studied and reviewed, there are practically no kinetic data available on these important reactions due apparently to a lack of reasonable procedures for assay of the reaction systems.

Because of the important theoretical, physiological, and industrial implications of these reactions, it appeared useful to have kinetic data on them and concomitantly thus to develop a method for obtaining such data.

The specific problem selected for study from this broad area was the measurement of reaction rates prevailing in the homogeneous system: cellobiose-1 molar sodium hydroxide-water at 22°C. and to derive the related rate constants from the reaction rate expressions and then to assess current understanding of these reactions in light of the kinetic data obtained.

ANALYSIS OF THE PROBLEM

Carbohydrate materials undergo both physical and chemical changes in aqueous alkaline solutions. If the material is soluble, then changes involving epimerization, isomerization, and other chemical reactions are expected. If the material and the solution form a heterogeneous system, then additional changes such as swelling and dissolution are also possible.

In this thesis and in apparent accord with current usage, the term "transformation" will be used to describe, in a collective sense, epimerization and isomerization reactions and the term "degradation" to mean other reactions undergone by carbohydrates in alkaline systems.

It thus appears that included in the steps necessary to solve the chosen research problem will be a delineation of the reactions occurring which will, in turn, require selection of techniques for the analysis of reaction mixtures. Then, depending on the complexity of the reactions found to occur, it will be necessary to devise an experimental design that will yield kinetic data sufficient to solve the appropriate reaction rate equations for rate constants. This, in turn, may lead to some careful selection of useful mathematical approaches to the problem of solving the rate equations.

Undoubtedly, the ensuing research program will proceed in a synergetic way, each advance shaping and reshaping the next. The first move is, of course, a search of the literature for information pertinent to the above probable steps.

LITERATURE REVIEW

The effects of alkaline solutions on various carbohydrates have been and are being studied extensively. The reviews in 1958 by Whistler and BeMiller (1) on the alkaline degradation of polysaccharides and by Speck (2) on the Lobry de Bruyn-Alberda van Ekenstein transformation, together with that in 1957 by Sowden (3) on the saccharinic acids and the 1960 review by Meller (4) on the alkaline degradation of cellulose provide a good introductory background to the presently held understanding of the alkaline degradation of carbohydrates. In 1955, McBurney (5) reviewed all aspects of the degradation of cellulose. A short review in 1963 by Richards (6) gives some information on related analytical procedures.

There is considerable evidence that the rates of degradative reactions proceeding in heterogeneous systems are controlled by transport phenomena. This evidence was reviewed in 1955 by McBurney (5) and in 1950 by Timell (7).

The oxidative degradation of cellulose in the presence of molecular oxygen (4, 5) and the relative stability of cellulose in a system free of oxygen (8) as well as the apparently catalytic effect on oxidative degradation from iron (9), manganese (10), and ultraviolet and daylight (5) have been reviewed.

The chain shortening and chain modification known to occur in cellulose under degradative attack by aqueous alkaline solutions in the absence of molecular oxygen and various metallic ions are presently considered to be the result of some combination of a peeling, a stopping, and a cleavage reaction. Each of these will now be discussed.

THE PEELING REACTION

In this reaction, it is pictured that the remainder of the chain is eliminated as an anion from the reducing end monomer which becomes the cation in a heterolytic

fission of the bond between the glycosidic oxygen and C4 of the modified reducing end monomer. Addition of a proton to the chain anion reestablishes the new end monomer as a reducing monomer, and thus it becomes available for a repetition of the peeling reaction. The peeled cation combines with an hydroxyl ion and then by a benzilic acid-type rearrangement is converted to the anion of either α - or β -D-glucoisosaccharinic acid. The elimination of the chain anion is considered to proceed by the β -alkoxy-carbonyl elimination mechanism as perhaps first suggested in 1907 by Nef (11) and as shown to be applicable by the now generally accepted proposals involving consecutive electron displacement as discussed in 1944 by Isbell (12). A possible sequence for a peeling reaction based on that given by Whistler and BeMiller (1) is outlined in Fig. 1. As both a peeling reaction and a stopping reaction probably begin with a Lobry de Bruyn-Alberda van Ekenstein transformation, the possible paths of some of those transformations are given in Fig. 2.

The 1953-57 studies by Kenner and associates (13-27) with dilute lime water solutions free of oxygen at 25°C. provided much evidence for the peeling reaction. They followed the rather slow reactions (up to 600 hours) by paper chromatography and the expected gradual appearance of various carbohydrates and saccharinic acids from a variety of carbohydrates was demonstrated. In some of the work, quantitative separations or analyses were done. Their work (18) on laminaribiose and turanose showed essentially the same rate of degradation for these disaccharides to meta-saccharinic acids. These findings led to the suggestion of a modified degradation mechanism as shown in Fig. 3. involving a common bivalent ion (IIA in Fig. 3) in the degradation of these 1 \rightarrow 3 linked disaccharides. This bivalent anion concept was then extended to include various trivalent anions (IIA³ and IIA⁴ in Fig. 3) in explanation of their finding (16) of mainly D-gluco-saccharinic in the degradations of 1-O-methylfructose, glucose, and fructose in dilute lime water, whereas Nef (28) had found mainly D-gluco-metasaccharinic acids and some D-glucoisosaccharinic

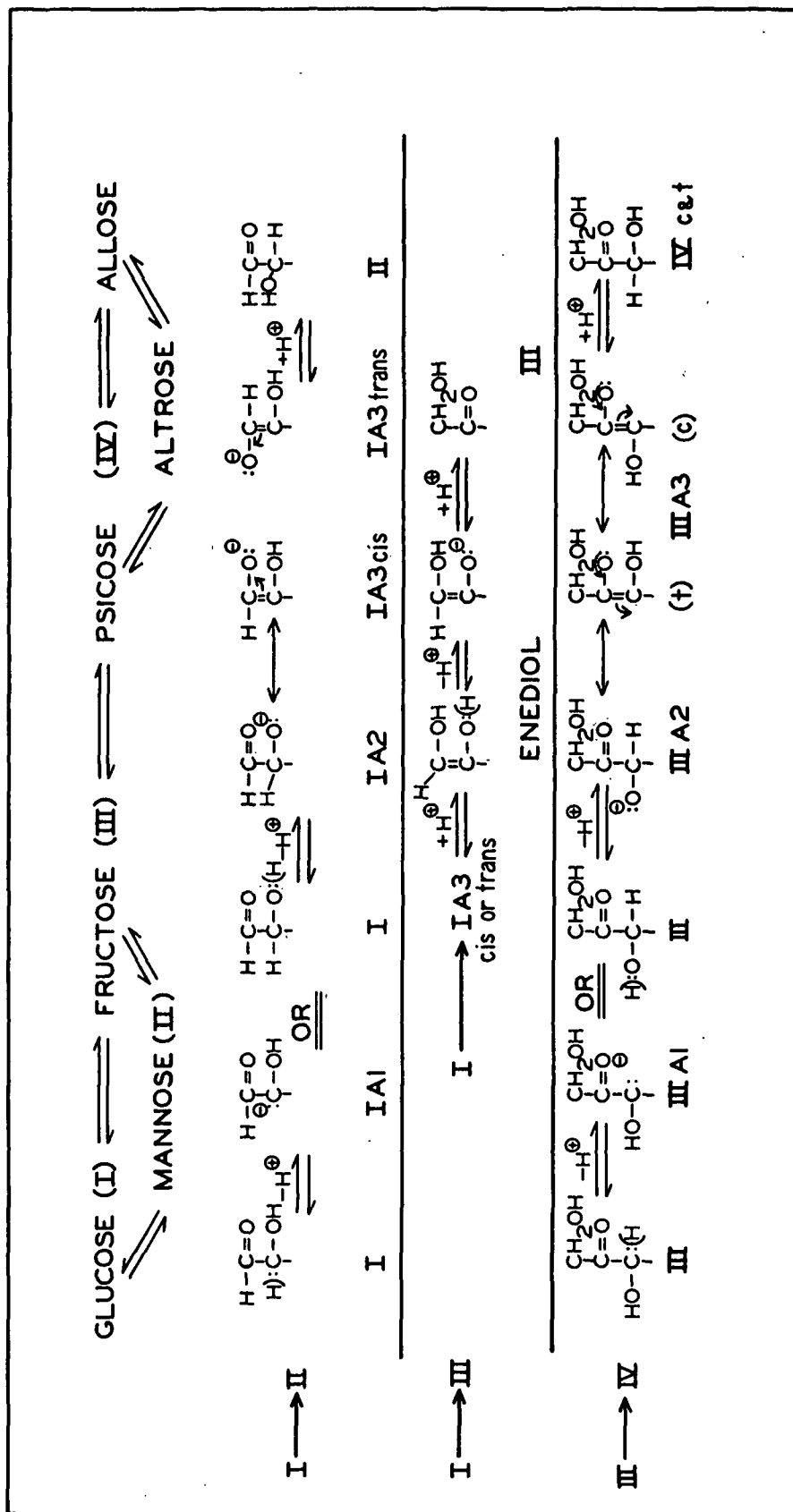


Figure 1. Possible Lobry de Bruin-Alberda van Ekenstein Transformations

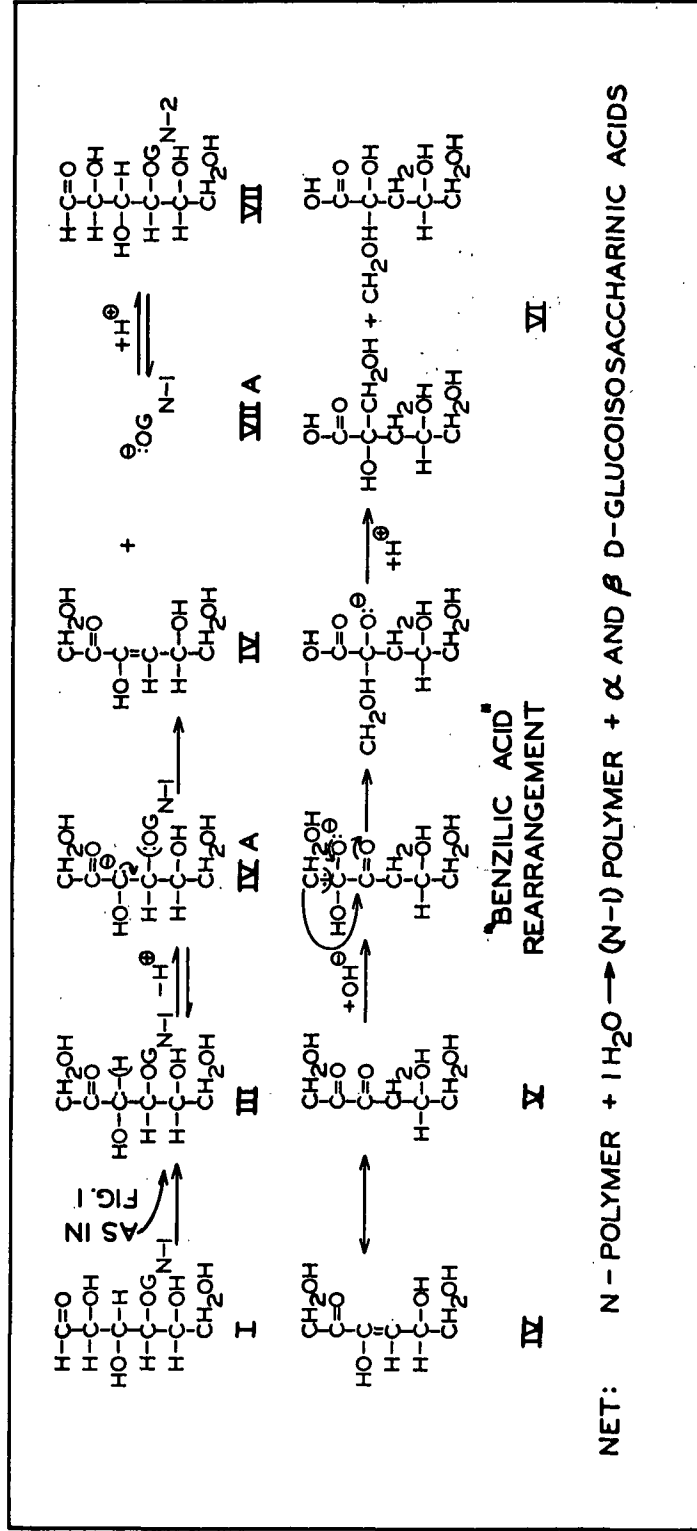


Figure 2. Possible Mechanism of the Peeling Reaction

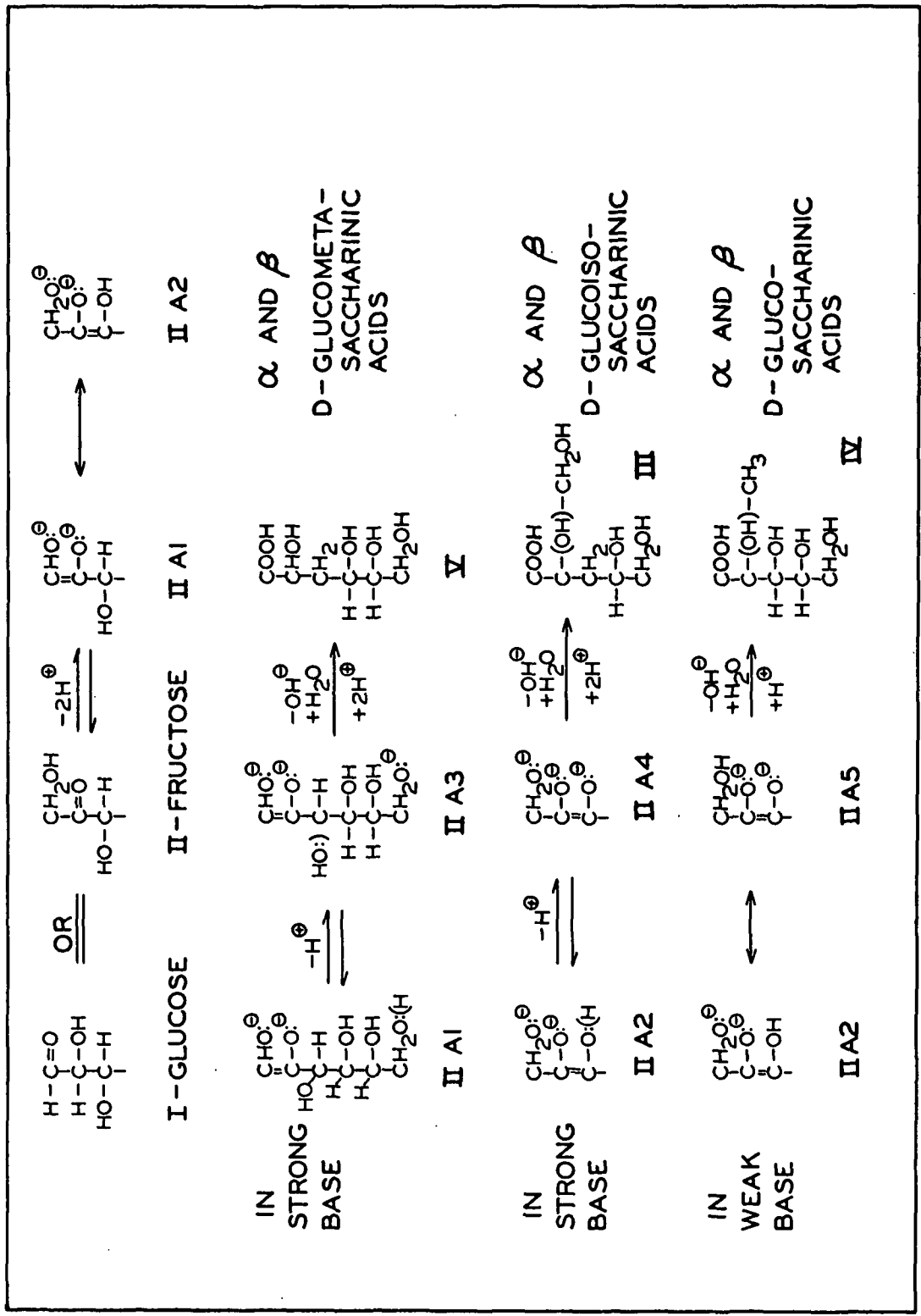


Figure 3. Saccharinic Acid Formation via Kenner (18) Schemes

acids from the degradation of glucose in 8M in sodium hydroxide. The plausibility of the trivalent ion concept was argued on the work of Urban and Williams (29) who had reported and evaluated a third acidic dissociation constant for glucose at pH 13.6. These various findings indicate that the peeling reaction mechanism is probably concentration dependent, though it should be noted that Kenner and Richards (16) and Nef (28) used different basic cations, thus suggesting that the mechanism of the peeling reaction may be also cation dependent. The cation dependency of degradation reactions is supported by other studies as discussed by Whistler and BeMiller (1).

Further insight into the proposed peeling reaction is available from the studies in 1957 by Richards and Sephton (30) on the soluble products from the action of sodium hydroxide solutions on cotton cellulose. Using 0.5M sodium hydroxide at 100°C., they found about 44% of the recovered carbon present as α - and β -D-glucoisosaccharinic acids and their lactones, about 8% as formic acid, 4% as lactic acid, and the remainder included less than 2% as acetic acid and a dozen or more compounds which were either tentatively or not identified. The established presence of these fragment acids is important in connection with the studies of Sowden and Kvenne (31) in 1953 with glucose and fructose bearing labeled C1 carbons. Among the products of alkaline degradation of each of these sugars they found glucosaccharinic acids with the labeled carbon in the methyl group as would be expected from the IIA2 to IV sequence of Fig. 3; but also they found the glucosaccharinic acid with the labeled carbon at C2 of the acid, which it was argued could arise only if the acid was formed via the condensation of three carbon fragments derived from a C3-C4 cleavage of the C1 labeled hexose in such a way that a C3 labeled hexose was formed and which then was converted to a C2 labeled glucosaccharinic acid by the mechanisms of, say, IIA2 to IV of Fig. 3. Thus it appears saccharinic acids may arise from the aldol condensation of fragments.

In 1958, Machell and Richards (32) reported degradation studies on amylose and compared the results with the previous studies on cellulose by Richards and Sephton (30). They found essentially the same soluble degradation products from amylose and cellulose though somewhat surprisingly, the rates were faster in the heterogeneous cellulose system than in the homogeneous amylose system at the same temperature (100°C.) and sodium hydroxide concentration (0.5M). Of course, it is not possible to compare the concentrations of the carbohydrates in these two systems. This work indicates the possibility of using $\alpha(1\rightarrow4)$ anhydroglucose polymers in homogeneous systems to gain qualitative understanding of the degradative reactions for $\beta(1\rightarrow4)$ anhydroglucose polymers. The studies of Green (33, 34) established the presence of various saccharinic acids in kraft black liquors through positive identifications as anilides, and thus added to the evidence for a peeling reaction.

It is conceivable that a peeling reaction could proceed in one of two distinct ways. The peeling could be a one-monomer-at-a-time, stepwise process, random with respect to all the chains with reducing ends. The other possibility is a rapid chain-type depolymerization of selected chains which is initiated by activation of certain chain ends and then rapidly propagated along that chain until either the chain is consumed or the propagation stopped for some reason before the chain is consumed.

Kenner and associates definitely subscribe to the stepwise viewpoint, and their work on cellotetraose (21) in which they reported the progressive appearance of cellotriase and cellobiose would seem to support their view. Collier (35) agrees completely with Kenner's views and offers supporting kinetic data from degradation studies in homogeneous systems. On the other hand, Samuelson and his associates (36), who have done considerable study on the degradation of cellulose, hold the chain reaction view very strongly. For example, Samuelson and Wennerblom (37) give drawings depicting the rapid disappearance of whole chains and state,

"The mechanism of reaction (peeling) must be assumed to involve some sort of a chain reaction." Then again, Stockman and associates (38) differ with their countrymen and support the stepwise suggestions of Kenner.

Whether a peeling reaction is stepwise or chain is probably of little consequence when loss in weight of product is the main or only concern. However, the shape of the chain length distribution curve of a product for a given weight loss through degradation would appear to depend significantly on whether a peeling reaction was stepwise or chain. The shape of this distribution curve is frequently very important. Then again, a stepwise process may prevail under some reaction conditions and a chain under others.

THE STOPPING REACTION

In this reaction, it is pictured that the reducing end unit is not peeled, but instead is converted and retained as either an α - or β -D-glucometasaccharinic acid unit through the possible sequences reviewed by Whistler and BeMiller (1) and outlined in Fig. 4.

Much evidence for a stopping reaction rests on the frequently observed event that in many cases degradation proceeds to a standstill with the production of an "alkali-stable" carbohydrate remainder. It was early observed that the reducing capacity of material under degradative attack gradually diminished to essentially zero, and the acidic properties gradually increased as the stable condition was approached. Then the glycosidation studies of Reeves, *et al.* (39) in 1946 and those of Meller (40, 41) in 1952 and 1953 showed that glycosidation, oxidation, or reduction of the reducing function rendered the material relatively stable to degradation. In 1954, Richtzenhain and Abrahamsson (42) showed that cellulose lost no further weight after 8 hours or so when boiled in 1M sodium hydroxide, and they suggested the possibility that saccharinic acid formation at the reducing

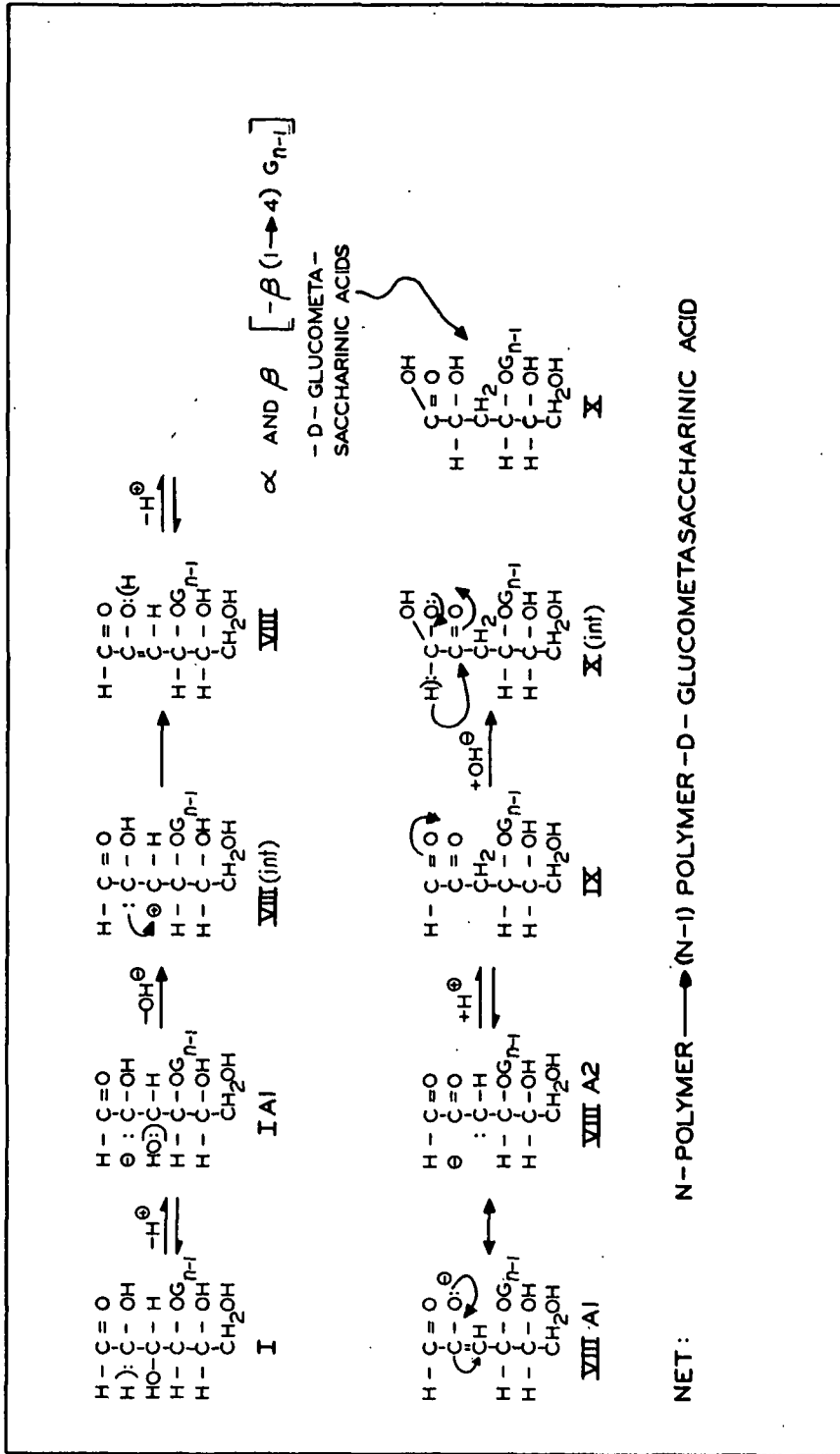


Figure 4. Possible Mechanism of the Stopping Reaction

ends of chains was the stabilizing factor. In 1957, Machell and Richards (43) made an alkali-stable material by boiling a cotton hydrocellulose in 0.5M sodium hydroxide and then isolated and identified α - and β -glucometasaccharinolactones via their strychnine salts from the acid hydrolyzate of the stable material. On this evidence, they proposed a stopping reaction based on the formation of a nonpeelable metasaccharinic acid, as shown in Fig. 4. Machell and Richards (32) showed in 1958 that the degradation of soluble amylose in 0.5M sodium hydroxide at 100°C. proceeded to a 40% stable remainder in about 20 hours, from which they identified by paper chromatography α - and β -D-glucometasaccharinic acids, thus supporting the stopping reaction mechanism they had proposed the previous year.

In connection with a stopping reaction, MacLaurin (44) found that a stable remainder of about 94% yield was obtained in 10 hours from a high alpha cotton cellulose which had been suspended in 1M sodium hydroxide at 100°C. Both the 100°C. stable product and fresh cotton cellulose gave a stable remainder of some 94% yield in 10 hours when suspended in 1M sodium hydroxide at 130°C. However, neither the 100°C. stable product, the fresh cotton cellulose, nor a hydrocellulose prepared from the cotton cellulose gave any indication of forming a stable remainder when suspended in 1M sodium hydroxide at 175°C., even though the reaction was continued for over 40 hours at which time yields were under 25%. All the 175°C. data showed that after an initial faster rate period, the weight loss rate followed a first-order-type reaction with the same rate constant for each of the three materials. While complete interpretation of these observations is probably not possible at present because of the heterogeneous systems employed, it does seem possible to conclude that:

(a) The findings from the 100°C. and 130°C. experiments support the concepts of a peeling and a stopping reaction. While the observed stabilization at these temperatures may have been due in part to the stoppage of chemical reactions because

of the physical stoppage of mass transfer, the existence of stopped ends at inter-phase boundaries seems most probable.

(b) The findings from the 175°C. experiments support the concepts of a peeling reaction and either a glucosidic or glycosidic cleavage or no stopping reaction.

THE CLEAVAGE REACTION

Firm experimental data to support the concept of a hydrolytic cleavage of a pure glucosidic acetal linkage in a (1→4) anhydroglucose polymer do not seem to be available. Samuelson and associates (36, 37, 45) have carried out many experiments to show relationships between DP_N , DP_W , yield, and carboxyl content in heterogeneous systems. They conclude that this cleavage does occur, and further, they calculate that some 50-60 monomers are peeled for every cleavage. Their primary experimental data are rarely included and, hence, it is difficult to analyze their conclusions. However, because of the known difficulties in attempting to "count" chain molecules in distributed heterogeneous systems and the difficulties of carboxyl group determinations on heterogeneous material, and the lack of evidence that a carboxyl is present at each chain end, it is concluded that the existence of a cleavage reaction of a glucosidic acetal linkage has yet to be established under typical reaction conditions. As discussed above in connection with the stopping reaction, it seems more logical to predict a stopped end glycosidic cleavage than a pure glucosidic cleavage in the body of a chain.

FURTHER PAPERS OF RELATED INTEREST

Strocchi and Gliozzi (46) considered the aldo-keto isomerization reaction in the D-glucose--D-fructose--D-mannose system and reported some kinetic data in 1953. The mechanisms of saccharinic acid formation were discussed by Machell and Richards

(47-50) in 1960, and a synthesis of phosphorylated glucometasaccharinic acid was reported by Lewak and Szabo (51). The nature of the saccharinic acids derived from the alkaline degradation of D-glucose, D-fructose, and D-mannose was presented by Feast, Lindberg, and Theander (52) in 1965. A paper by Ishiza, Lindberg, and Theander (53) in 1967 gave further similar data.

The preparation of 4-O-D-glucosyl-D-mannose from cellobial was reported in 1921 by Bergmann and Schotte (54), in 1926 from cellobioseoctaacetate by Brauns (55), in 1930 by a modification of that route by Isbell (56), in 1946 by Danilov and Pastukhov (57) from cellobiose treated with calcium hydroxide solution, and in 1968 by Alexander (58) through an enzyme induced coupling between α -D-glucose-1-phosphate and D-mannose.

PAPERS RELATED TO ANALYTICAL ASSAY OF REACTION MIXTURES

Corbett (59) gave details of the separation of disaccharides from monosaccharides on charcoal-celite columns based on the earlier work of Whistler and Durso (60). Stefanovic (61) made separations and gravimetric recoveries of glucose, maltose, and raffinose on charcoal-aluminum oxide columns. An improved quantitative paper chromatography separation and elution of glucose from glucose-cellobiose mixtures was given by Kraske (62). The separation of cellobiose on charcoal columns from complex mixtures obtained during enzymatic synthesis has been reported by Ramamurti and Jackson (63). A heat of dilution modification of volumetric dichromate oxidation was developed by Launer and Tomimatsu (64, 65) for the quantitative determination of carbohydrates.

The well-established procedure for detecting the location of carbohydrate materials on paper chromatograms by dipping the sheet in alcoholic silver nitrate solution, etc., was first developed by Trevelyan, Proctor, and Harrison (66).

The p-anisidine spray of Hough, Jones, and Wadman (67) for carbohydrates with a reducing function and the anthrone spray of Johanson (68) for ketoses are of probable interest.

CONCLUSIONS FROM THE LITERATURE REVIEW

Guided by the preceding literature review, it was concluded that an experimental system designed toward the research objectives of the problem must be homogeneous, free of molecular oxygen and certain metal ions, and the reactions should be carried out in the dark. While the reactions of interest appeared to be cation dependent, aqueous sodium hydroxide was chosen as the base because of practical interest despite the recognized problem of preparing and maintaining such solutions in a "carbonate free" form. Because of the apparent complexity of the reactions likely to be encountered, it appeared logical to select cellobiose, the simplest possible $\beta(1\rightarrow4)$ glucan, as the molecule best suited for these studies. In addition, this molecule eliminated the possibility of an internal glucosidic bond cleavage reaction as discussed above. It also eliminated the possibility and necessity of considering the alternatives of a chain or stepwise peeling reaction. None of these built-in simplifications appeared to be possible sources of an a priori bias in the study. Furthermore, cellobiose was available commercially in ample supply and in essentially pure form as a crystalline compound of known physical properties and at a reasonable cost. Thus, the homogeneous system: cellobiose-sodium hydroxide-water was chosen for this study.

The literature review clearly showed the lack of kinetic data for the transformation and degradation reactions of $\beta(1\rightarrow4)$ glucans. Mention of the experimental difficulties of assay of the reaction systems involved was frequently encountered when lack of such kinetic data was discussed.

It was also evident from the review that there is a lively continuing interest in this area of carbohydrate chemistry.

EXPERIMENTAL RESULTS

As outlined earlier, the objective of this research was to obtain reaction rate constants prevailing in a homogeneous cellobiose--sodium hydroxide--water system and to consider the implications of these rate constants in terms of the related reactions.

In this study, it was established, experimentally, that for the chosen reaction conditions the neutral carbohydrates arising from cellobiose at any time during the reaction in any amount greater than about 0.5% by weight of the starting cellobiose were limited to cellobiulose, 4-O- β -D-glucosyl-D-mannose, D-glucose, D-fructose, and D-mannose. This conclusion rested on the nonappearance of other significant peaks on chromatograms and the particular determination of the location of D-allose* and D-altrose* on control chromatograms. This conclusion is well supported by theoretical considerations and the experimental observations of others. Then, of course, the conclusion is confirmed, a post-eriori, by the interlocking agreement of the reaction rate constants derived from the postulated reaction scheme based on these carbohydrates.

Each of the carbohydrates shown to be present in a cellobiose reaction system was then used as starting material in a series of reaction runs in which the concentration of each of them present after various reaction times was determined through resolution of a prepared sample of the reaction system by column chromatography and colorimetric analysis of the column effluent. A detailed description of the materials and solutions used is given in Appendix I and of the apparatus and procedures used in Appendix II. In brief summary, the procedures used were as follows.

*These materials were an appreciated gift from Dr. John W. Green.

The reactions were carried out in screw-capped pyrex tubes using 25 ml. of one molar sodium hydroxide solution and 1 ml. of the carbohydrate solution. In reaction systems for monomers, the starting molarity of the carbohydrate was about 0.002 and for the disaccharides about 0.001. These relative concentrations assured essentially no change in hydroxyl concentration during the consumption of at least half of the starting material. These carbohydrate concentrations were chosen for the further reason that they could be used with half and even quarter molar base, with again little effect on base concentration during a useful first part of the reaction. This latter was in anticipation of experiments to examine the effect(s) of base concentration. The reaction was quenched with hydrochloric acid. An aliquot from the quenched system was prepared for chromatography by admixture with a buffered boric acid solution. The carbohydrates were resolved in 8-9 hours by anion-exchange resin column chromatography. The column effluent was monitored continuously with a Technicon Auto-Analyzer system using an orcinol/sulfuric acid reagent for colorimetric analysis. The analytical results were recorded on a quantitative strip chart chromatogram. Figure 5 shows Xerox copies of a control chromatogram of a prepared mixture of the six carbohydrates of interest arising in a cellobiose system and of a chromatogram from a 22-hour reaction with cellobiose. These chromatograms show the acceptable shape of the peaks and the clear-cut resolution of the several carbohydrates.

The complete chromatography data for all the reactions are given in Appendix III. This appendix also includes tables of the relative molarities of the various carbohydrates present in reaction systems at various reaction times. From these data, large-scale plots were constructed of relative molarity vs. time for each reaction run series. These plots were used in obtaining first approximate values for the reaction rate constants.

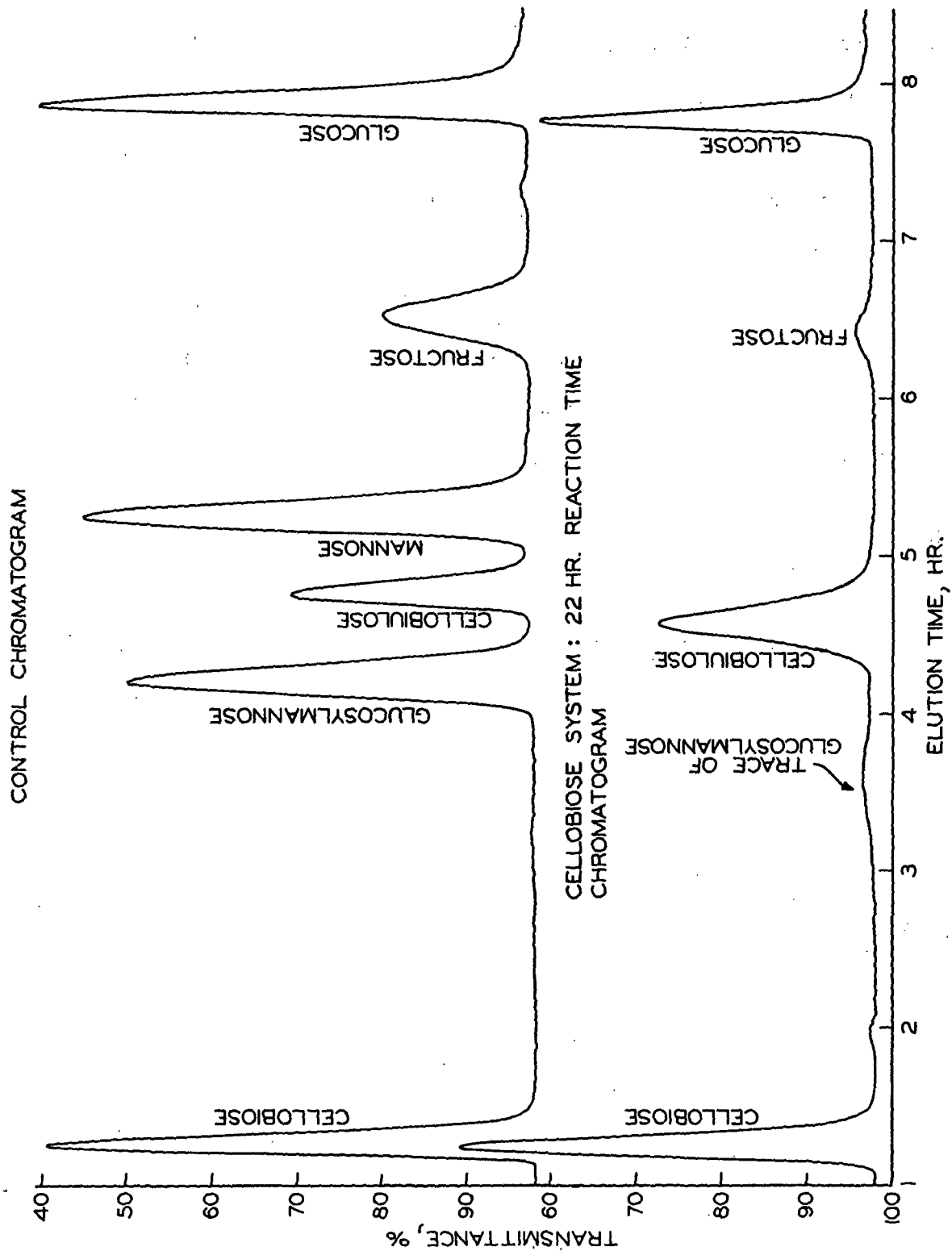


Figure 5. Typical Chromatograms

Based on the experimental evidence from the reaction runs and on theoretical considerations, the general case reaction scheme given in Fig. 6 was established. The appropriate reaction rate equations were then written for that scheme. From these equations initial approximate values for the rate constants were obtained by a combination of graphical and numerical procedures as described in Appendix IV. The rate constants related to the monomers alone were obtained first. Then these were refined and used in the solutions of the equations for the disaccharide systems and the peeling reaction rate constant for the reaction bridging the disaccharide to the monomer system. Initial values of rate constants were refined through a reiterative solution process on a computer until the rate equation curves (concentration vs. time) plotted by the computer from a given set of rate constants were a visual best fit with the experimental data. The computer program is described in Appendix V. The final values for the rate constants thus derived are shown in Fig. 6 and are listed in Table I.

Xerox copies of the concentration vs. time curves plotted by the computer using the program given in Appendix V and the rate constants listed in Table I are given in Fig. 7-13.

Figure 7 is the complete plot for the cellobiose system. A side-by-side comparison of the three complete disaccharide systems is shown in Fig. 8. In Fig. 9 are shown plots of the products, other than the carbohydrates determined, arising from each of the six carbohydrates as they occur in the three disaccharide systems. Figures 10 and 11 show separate side-by-side comparisons of the disaccharides alone and the monomers alone from each of the three disaccharide systems. In these two plots, the experimental data points are superimposed on the plots. The monomer systems with data points are shown in Fig. 12, and the products from these monomers, not determined, are shown in Fig. 13. The validity of these

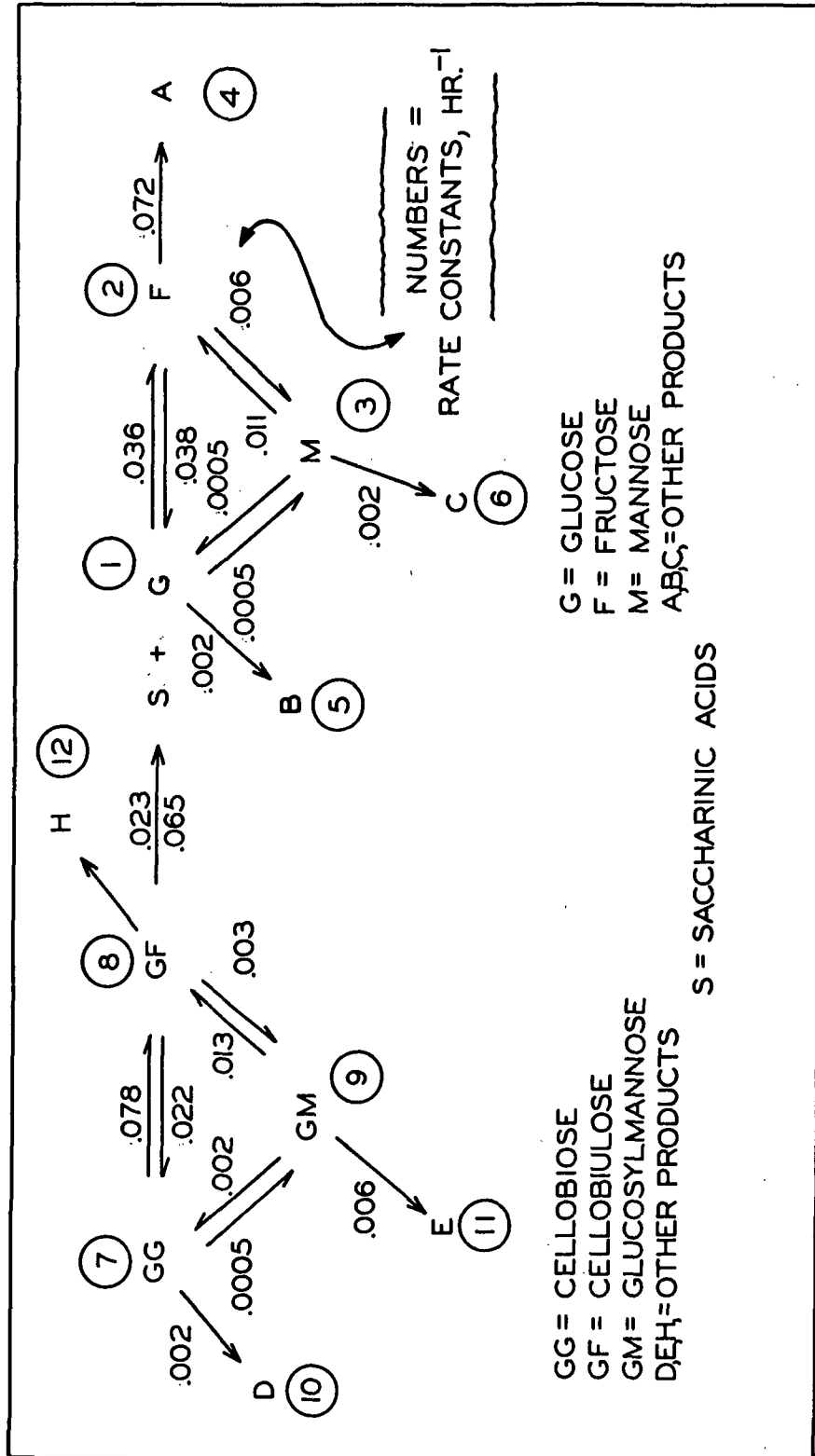


Figure 6. Cellobiose Reaction Scheme in 1 Molar Sodium Hydroxide at 22°C.

experimental results are discussed in the next section of this thesis along with inferences and conclusions it seems possible to draw from them.

TABLE I
RATE CONSTANTS IN THE SYSTEM 0.001M CELLOBIOSE
IN 1M SODIUM HYDROXIDE AT 22°C.

Reaction Pathway	Rate Constant	Rate Constant Value, hr. ⁻¹
G--F	\underline{k}_{12}	0.036 ± 0.001
G--M	\underline{k}_{13}	0.0005 ± 0.0002
G--B	\underline{k}_{15}	0.002 ± 0.0005
F--G	\underline{k}_{21}	0.038 ± 0.001
F--M	\underline{k}_{23}	0.006 ± 0.0005
F--A	\underline{k}_{24}	0.072 ± 0.001
M--G	\underline{k}_{31}	0.0005 ± 0.0002
M--F	\underline{k}_{32}	0.011 ± 0.0005
M--C	\underline{k}_{36}	0.002 ± 0.0005
GG--GF	\underline{k}_{78}	0.078 ± 0.001
GG--GM	\underline{k}_{79}	0.0005 ± 0.0002
GG--D	$\underline{k}_{7,10}$	0.002 ± 0.0005
GF--GG	\underline{k}_{87}	0.022 ± 0.0005
GF--GM	\underline{k}_{89}	0.003 ± 0.0005
GF--S+G	\underline{k}_{81}	0.065 ± 0.001
GF--H	$\underline{k}_{8,12}$	0.023 ± 0.001
GM--GG	\underline{k}_{97}	0.002 ± 0.0005
GM--GF	\underline{k}_{98}	0.013 ± 0.001
GM--E	$\underline{k}_{9,11}$	0.006 ± 0.0005

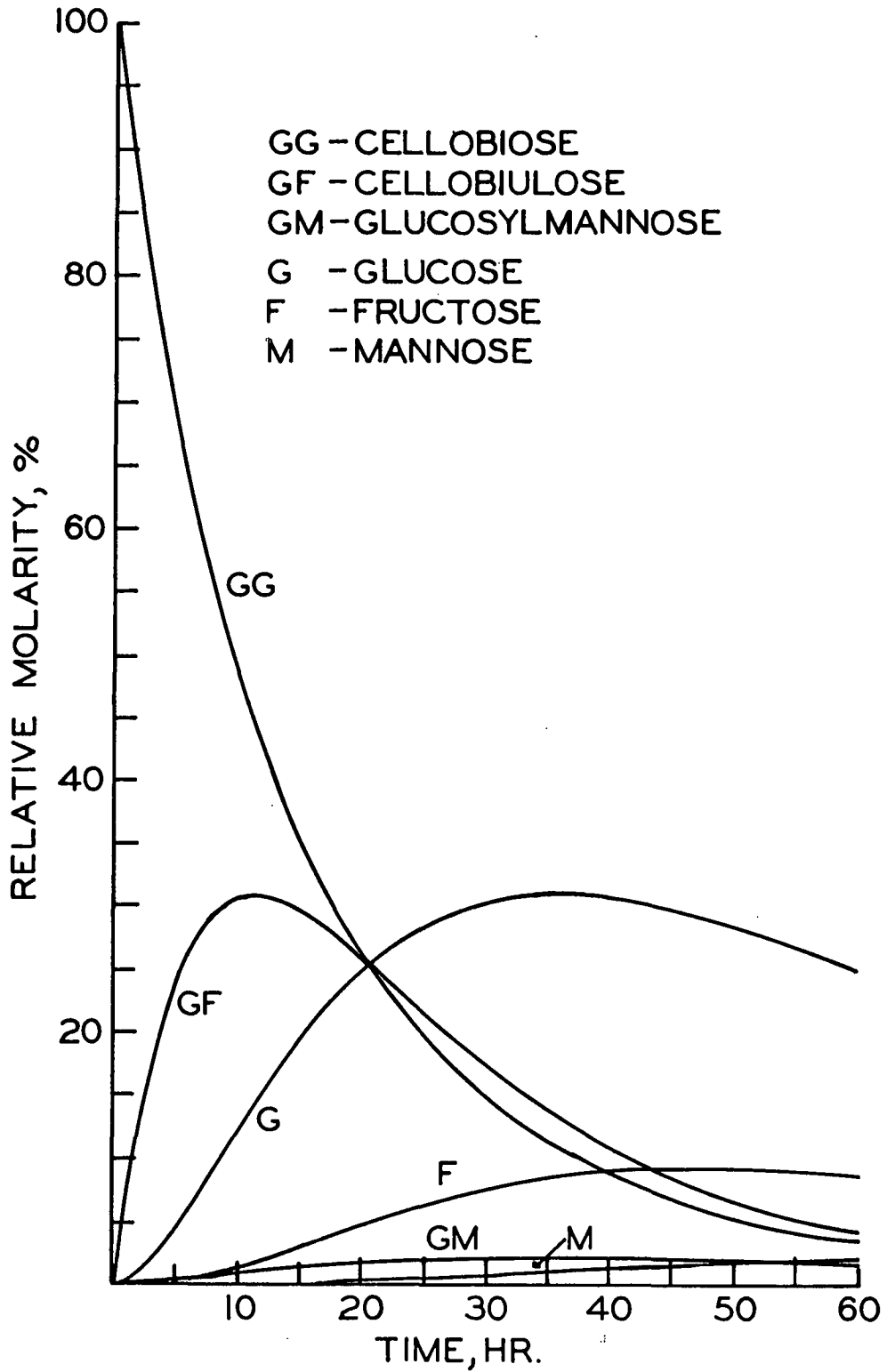


Figure 7. Cellobiose in 1M Sodium Hydroxide at 22°C.

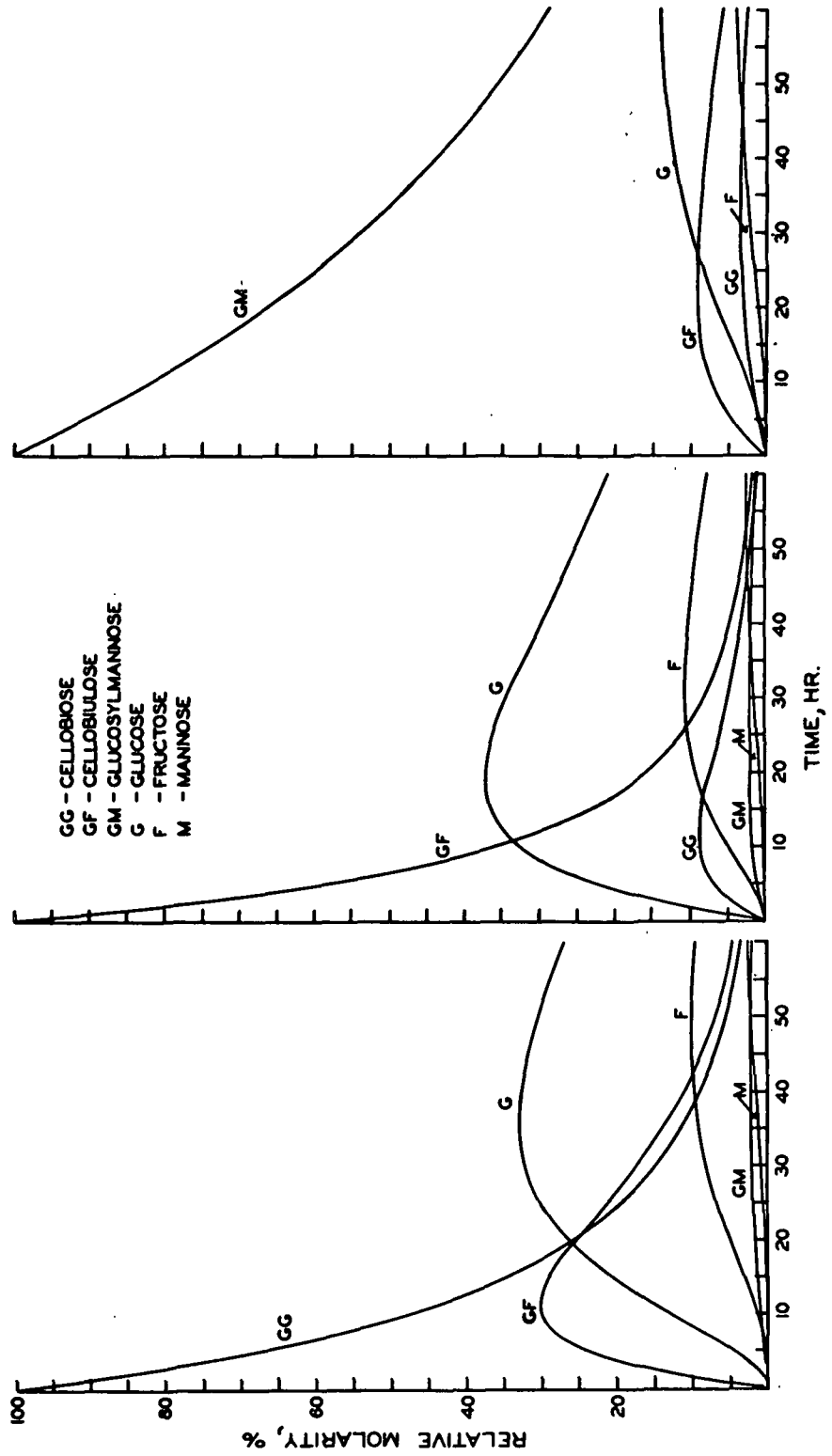


Figure 8. Disaccharide Systems-Carbohydrates in 1M Sodium Hydroxide at 22°C.

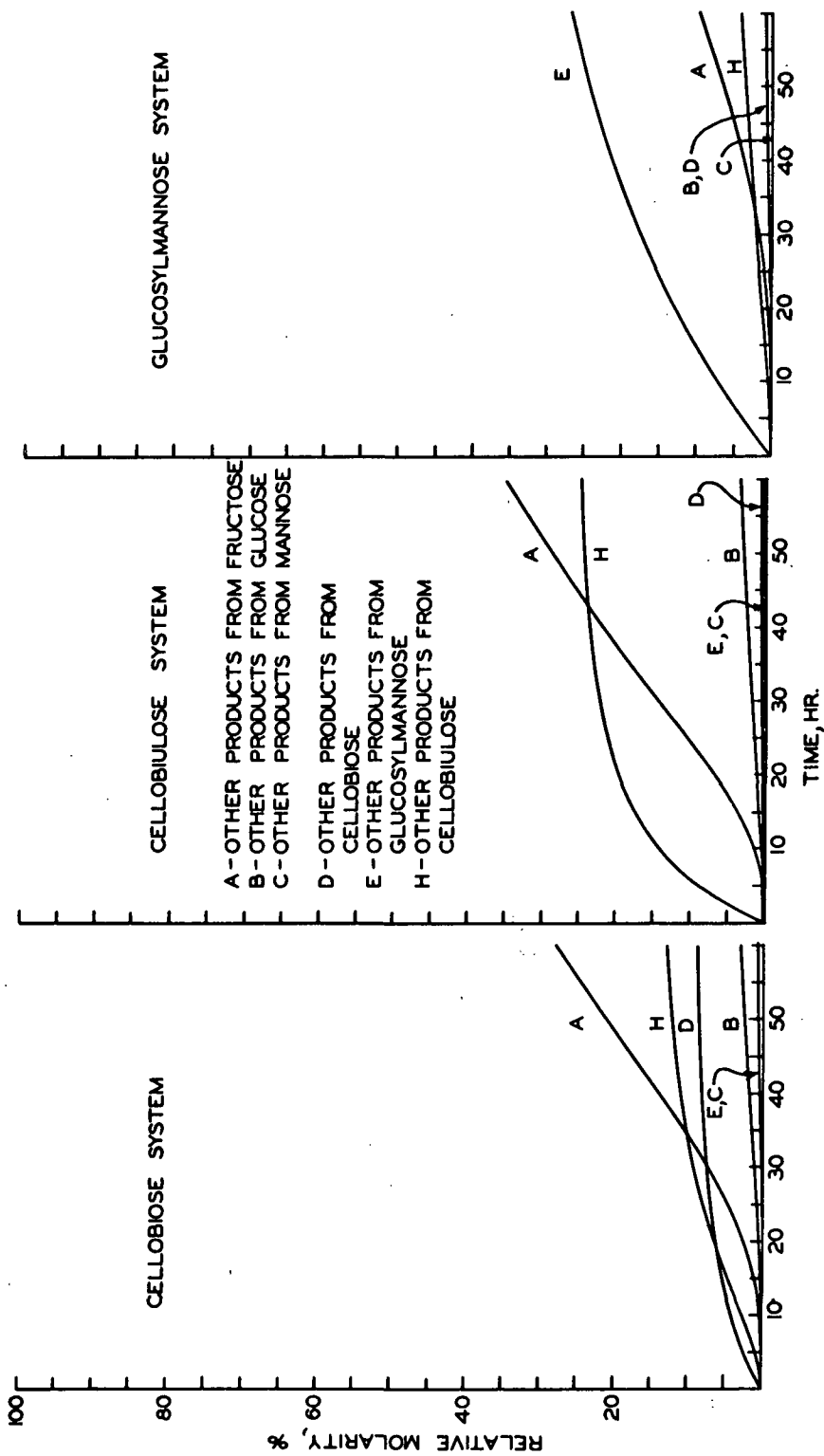


Figure 9. Disaccharide Systems - Other Products in 1M Sodium Hydroxide at 22°C.

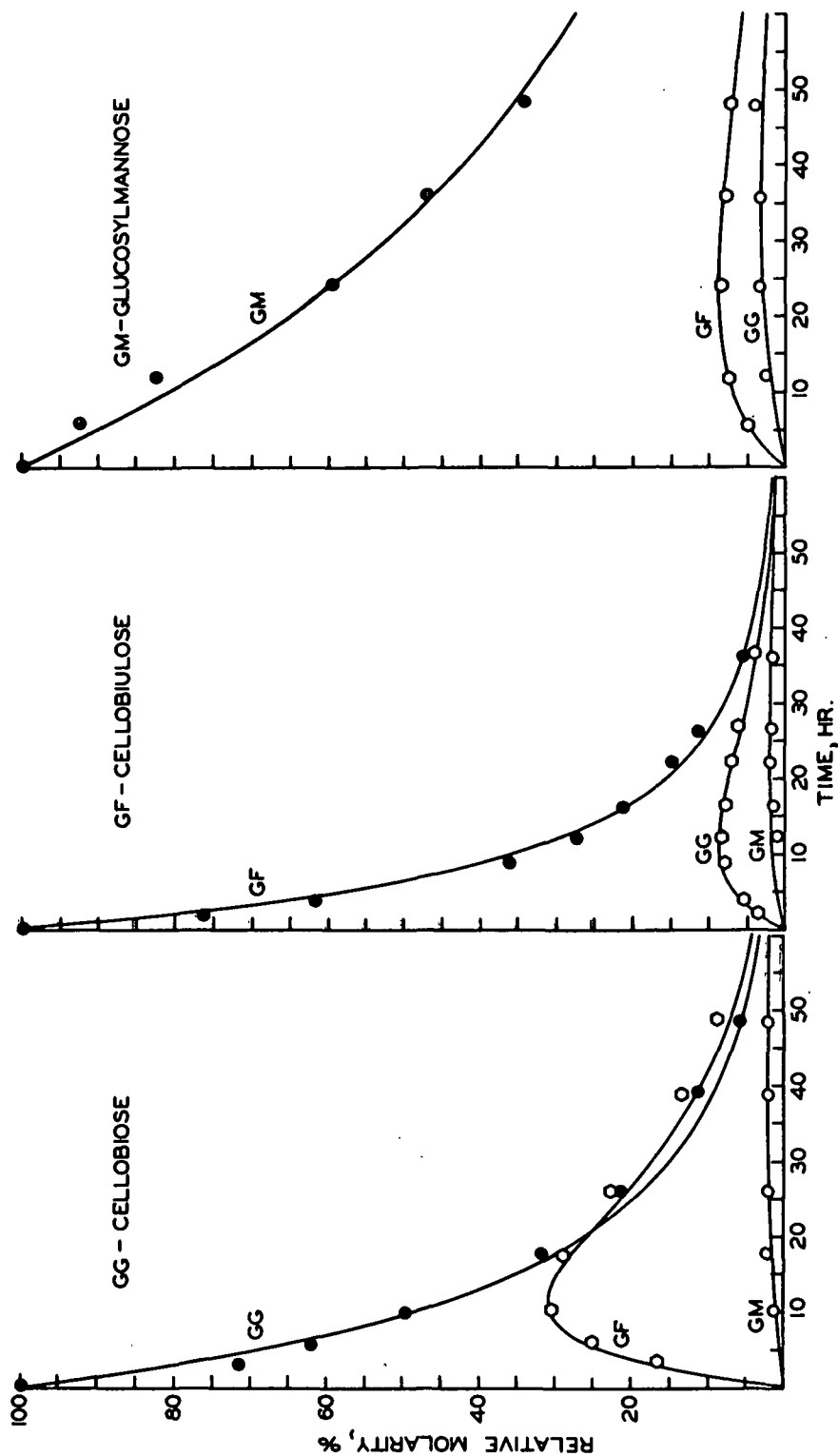


Figure 10. Disaccharide Systems - Disaccharides in 1M Sodium Hydroxide at 22°C.

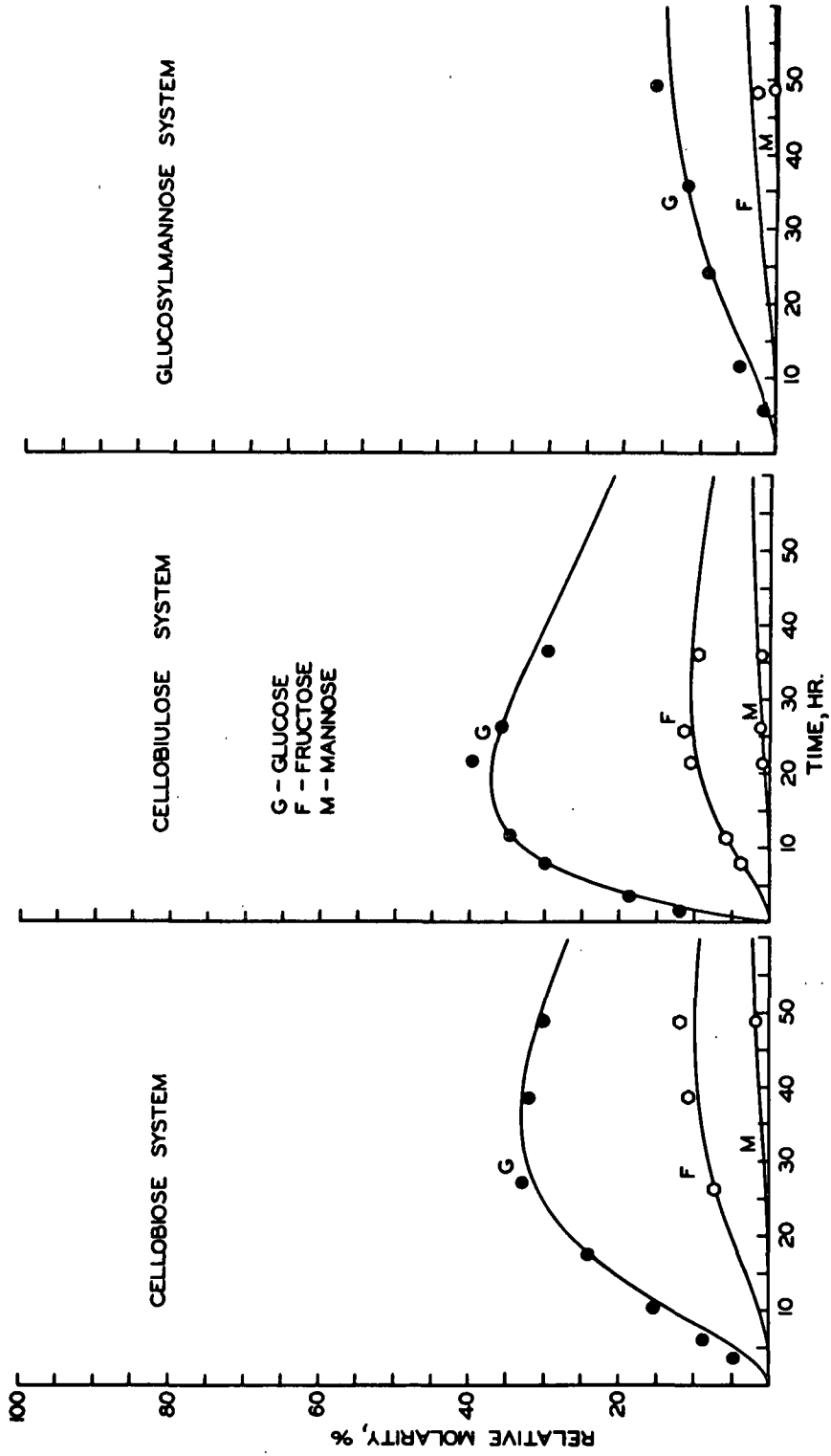


Figure 11. Disaccharide Systems - Monosaccharides in 1M Sodium Hydroxide at 22°C.

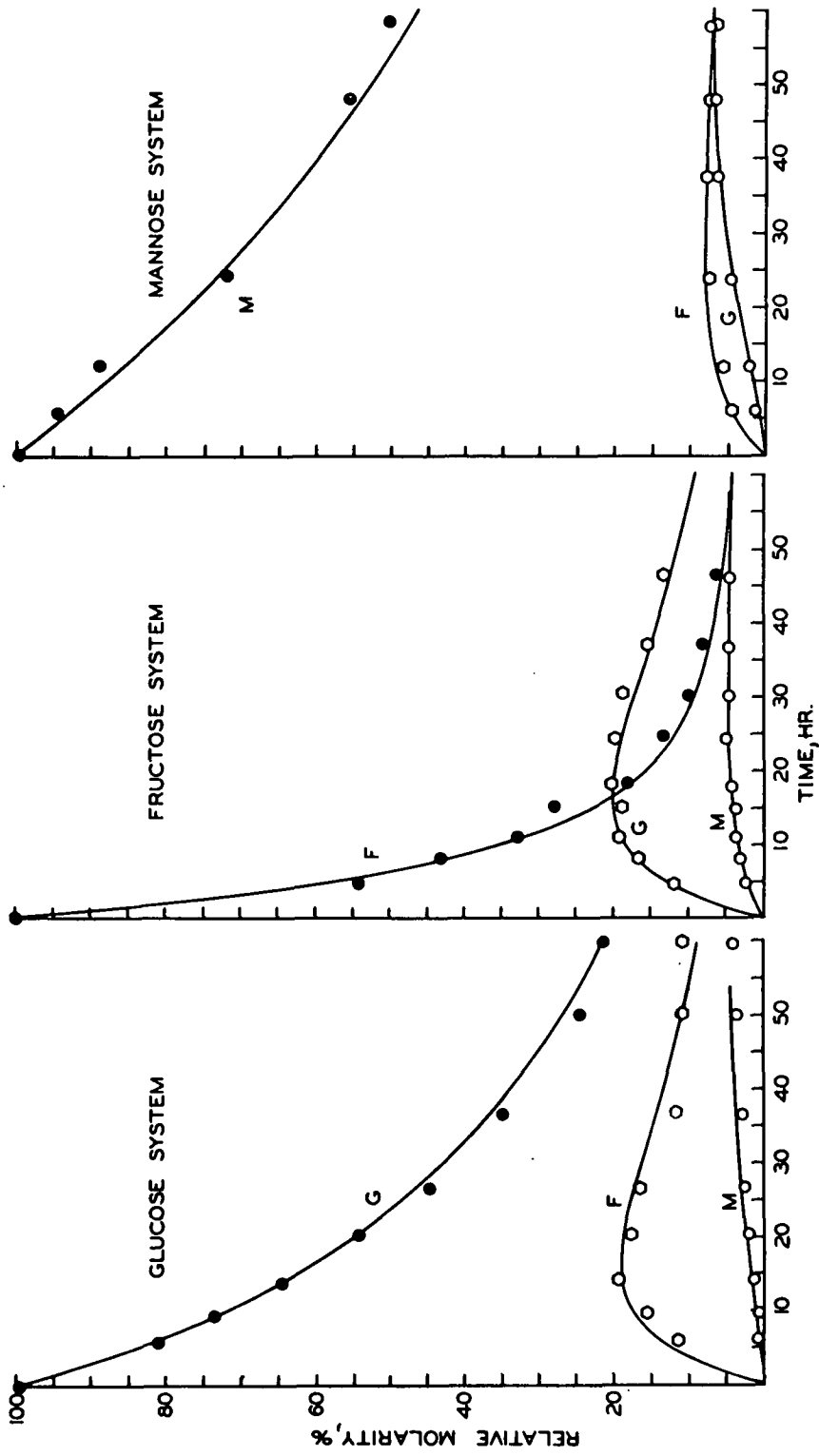


Figure 12. Monosaccharide Systems - Carbohydrates in 1M Sodium Hydroxide at 22°C.

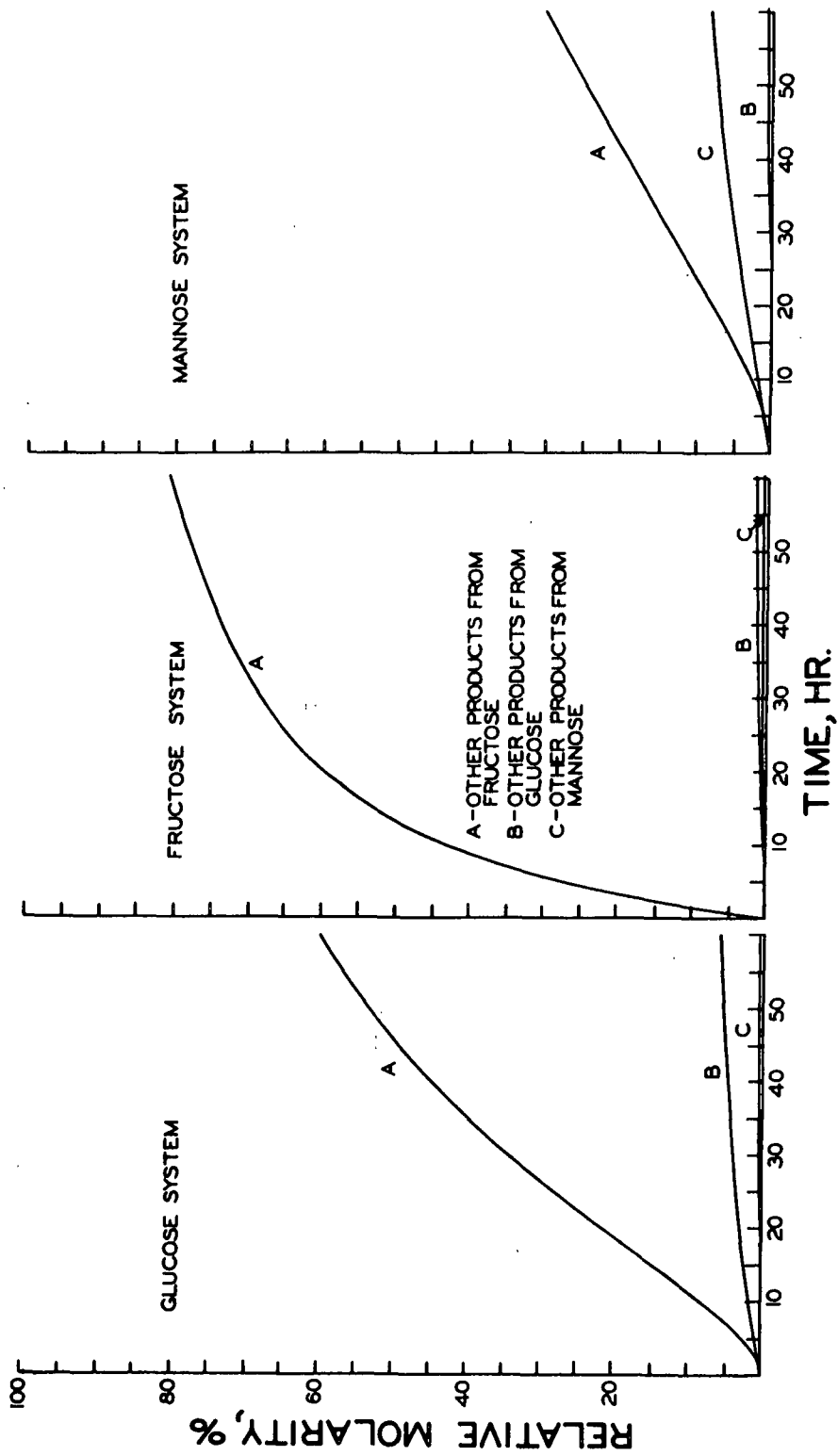


Figure 13. Monosaccharide Systems - Other Products in 1M Sodium Hydroxide at 22°C.

DISCUSSION OF THE EXPERIMENTAL RESULTS

THE EXPERIMENTAL DESIGN--AN EVALUATION

For some time in the earlier phases of this study the existence of mannose and glucosylmannose as reaction products from cellobiose was not established. Their existence was expected and accordingly careful search was made for them. It is noted that failure to establish the presence of mannose and glucosylmannose is now considered to be due to the relative insensitivity of quantitative paper chromatography in the detection of materials present in amounts below about 5% of the material present in greatest amount. Particularly is this so when the materials present in the small amounts run closely with those in great amounts. It was shown that the pairs cellobiulose-glucosylmannose and fructose-mannose were very difficult to separate on paper despite exploration with a wide range of solvents. Further, it is not possible using presently available developers and sprays to determine that both a ketose and an aldose are present at essentially the same point on a paper chromatogram. A ketose can be identified without interference from an aldose, by means of the anthrone spray (68), but the determination of an aldose without interference from a ketose with a similar R_f has not been accomplished. Thus, because of the small amounts of mannose and glucosylmannose in the reaction systems and the difficulty of separating them on paper chromatograms, they were not detected as long as paper partition chromatography was used to resolve the reaction mixtures. On these grounds (and others), this form of chromatography is considered to have serious limitations for studies of the type undertaken in this research. On somewhat similar grounds, separations on charcoal columns, based on the methods of White and Subers (69), proved unsatisfactory.

Trials with anion-exchange resin column chromatography, recently adapted to carbohydrate analysis by Kesler (70), showed a much greater sensitivity of detection, at least 1 in 200. Also, a greatly improved resolving power was available, due mainly to the flexibility of the elution process, through controllable variation of the elution gradient, the column temperature, and the elution rate. These more sensitive techniques soon established that mannose and glucosylmannose were present in small amounts in the reaction systems and, additionally, that other carbohydrates, theoretically possible through transformation reactions (2), were not present, at least in amounts greater than 0.5% of the starting material.

In retrospect, the experimental design evolved to abstract the reaction rate constants from the reactions occurring was adequate. Solving the "monomer triangle" first and then carrying those data into the "disaccharide triangle" and the peeling reaction bridge between the triangles, by moving in from every corner of each triangle, provided the means of separating the rate constants for the several simultaneous reversible reactions which occur. As far as can be determined, a reaction system of this nature has not been dealt with previously from a kinetic point of view.

ACCURACY AND PRECISION OF THE RATE CONSTANTS DATA

The overall accuracy of the reaction rate constants and the validity of their estimated precision, as given in Table I, is the cumulative result of combining the accuracy and precision of the basic chromatography data, the conversion of those data to relative molarities, and then the fitting of the experimental rate data with computed rate curves. Each step in this sequence will now be considered.

ACCURACY AND PRECISION OF THE CHROMATOGRAPHY DATA

The accuracy of the chromatography data depends on two factors: the purity of the carbohydrates used and their quantitative measurement. As discussed in

Appendix I, the chromatographic purity of the carbohydrates was established to at least 1 in 500. The least accurate operation in all the weighing and volumetric operations used was the measurement of the volume of the sample delivered by micropipet to the column for chromatography. Extensive gravimetric calibration of this type of pipet in these laboratories showed a delivery accuracy of at least ± 0.002 ml. for any volume in the range 0.100 to 2.000 ml., delivered from any portion of the total volume in the pipet. Thus, by using a sample volume of from 0.300 to 0.700 ml., an accuracy of at least 1 in 100 was obtained for this operation. In the use of the micropipet, extreme care had to be observed to eliminate entrainment of unobservable air bubbles at the Luer joint where the metal hypodermic needle was joined to the glass pipet barrel.

The precision of the chromatography data is measured by the scatter of the data of replicate runs. Because of the straight-line form of the absorbance vs. weight relationship, as discussed in Appendix III, it follows that the sensitivity and hence measurable precision of the chromatography data for a given carbohydrate will improve with an increase in the slope of this line. In this study, it was demonstrated that the shape and hence height of a chromatogram peak for a given quantity of material was determined by several factors which included: the amount of the carbohydrate chromatographed, the characteristics of the resin in the column, the concentration and pH gradients of the eluent, the column temperature, the rate of elution, the concentration of the orcinol/sulfuric acid reagent solution, and the ratio of the volumes of column effluent and reagent solution used for colorimetric analysis.

It was found that the optimum amount of carbohydrate was one that gave an absorbance value between 0.030 and 0.350. It appeared that the most important factor in resin characteristics was the range and distribution of particle size of the resin particles and the shape of the particles. Several resins were used

successfully in this study. However, the resin used to obtain the bulk of the data (Technicon 3/28/VI) had a narrower range of particle size at an apparently better average particle diameter and more nearly spherical particles than other resins available. Presumably, these factors contributed significantly to its better performance, compared with Bio-Rad AG1-X8, 325-400 mesh resin which is Dowex AG1, sized and prepared by the Bio-Rad Corporation of California. It is understood that the Technicon Corporation is actively pursuing the production of a resin equivalent to the 3/28/VI resin which at present has an experimental status.

In general, steeper elution gradients, higher column temperatures (70, 71), faster elution rates and higher orcinol concentration led to sharper peaks. However, stability while on the column of the materials being chromatographed (72) and their resolution may suffer when peak sharpening is pushed too far. A paper by Peterson and Sober (73) proved very helpful in considerations of gradient design. It provided the mathematical solution for determining the percent contribution from each chamber to the flow at any time from the last of a number of equal-volume chambers connected in series flow. By relating time of elution of a given carbohydrate to the approximate gradient concentration then leaving the column, it was possible to design gradients toward desired results. However, it was also evident that the pH of the eluent, and hence the ionic species present, was also an important factor in controlling the rate of movement of compounds along the resin column. In that connection, the studies by Ingri (74) provided useful information on the relationship between pH and ionic species containing boron in buffered boric acid systems.

In this study (see Table XIV, Appendix III), it was possible to select conditions so that a change in peak height of the smallest readable amount on the chart (about 0.001 absorbance for values up to 0.500 absorbance) was equivalent to less than

0.2 μg . of carbohydrate for all the carbohydrates except fructose where 0.001 absorbance was equivalent to just under 1.0 μg . of fructose. Thus, by selecting an amount of sample for chromatography containing 40 to 50 μg . of the key carbohydrate, the precision of the readability of the chromatogram for that carbohydrate became about 1 in 200.

An additional factor in the precision of the chromatography data was the overall stability of the chromatography--Auto-Analyzer system. This was the least acceptable link in the whole sequence. While a complete track-down and quantification of all the contributing factors was not made, it was easily established that the short life of the acidflex (synthetic rubber) tubes on the peristaltic proportioning pump, their changing performance during their life, and the lot-to-lot differences in these tubes contributed significantly to the run-to-run variability. From studies in this research and continuing studies in these laboratories, this system instability results in a precision (scatter about the best fit line) of about $\pm 2-3\%$ for single value determinations of weight of carbohydrate in a given sample. Of course, replication of data leads to average values of higher precision. In this study, all values are single values, unless otherwise shown as in the glucosylmannose data.

PRECISION OF THE CONVERSION OF THE CHROMATOGRAPHY DATA TO RELATIVE MOLARITY DATA

In addition to the variabilities noted in the preceding paragraphs, the precision of the converted data will also be adversely affected by long-term drift in calibrations. While this factor was not exhaustively studied, it appears from the interlocking fit of the concentration vs. time data between the various series of reaction runs which spread over a period of five months that precision was less affected by long-term factors than by short-term factors.

PRECISION OF THE COMPUTER CURVE-FITTING STEP

From many hours of incremental adjustment of computer plotted curves to the experimental data, it was empirically established that the estimated precisions of the rate constants as given in Table I are adequately liberal.

Thus; in summary, the rate constant data are considered to have accuracies of about $\pm 2\%$ and precisions as given in Table I.

CONCLUSIONS

At the beginning of this thesis, it was stated that the specific problem selected for study was to measure the reaction rates prevailing in the homogeneous systems: cellobiose-1M sodium hydroxide-water at 22°C. and then to derive the related rate constants from the reaction rate expressions and finally to assess current understanding of these reactions in light of the kinetic data obtained.

The reaction rates have been measured and the related rate constants obtained. In attaining these objectives, the disaccharide, cellobiulose, (4-O-β-D-glucosyl-D-fructose) was prepared by a modified procedure and another disaccharide, 4-O-β-D-glucosyl-D-mannose, was prepared by a modified more facile procedure with a significantly improved yield. It has been shown that the quantitative analysis of complex mixtures of disaccharides and monomers can be done with good accuracy and precision using ion-exchange resin column chromatography and colorimetric assay of the column effluent. Continuing experiments have indicated that more complex systems involving up to at least octaoses can be quantitatively analyzed by these same techniques. This capability would appear to be unique to these procedures, considering other possible alternatives presently available, such as gas and paper chromatography. A particular application of these analytical procedures is the determination of the chromatographic purity of carbohydrates (and undoubtedly other compounds) with an accuracy in the order of $\pm 0.1\%$. Such data, coupled with an ultimate analysis of the material, should be of help in determining its absolute purity.

In attaining solutions to the reaction rate equations, the application of a reiterative mechanical process, using a computer to solve an array of simultaneous differential equations, was demonstrated.*

*This application will be described in a paper (probably J. Chem. Ed.) by R. A. Holm and D. J. MacLaurin.

Present understanding of the actual mechanisms of the transformation and degradation reactions of the types shown to occur in the systems studied in this research appear to result largely from speculative inference. Actual experimental evidence in support of proposed reaction pathways is rather limited. There is though, as discussed in the Literature Review section of this thesis, considerable experimental evidence that the transformation reactions occur and that certain degradation reactions, particularly the "peeling" and the "stopping" reactions occur. The following paragraphs compare and discuss the present findings in terms of previous findings where such are available.

In the glucose-fructose-mannose system, the expected reversible transformation reactions involving changes at Carbons 1 and 2 have been observed for each of them. Transformation reactions involving other carbon atoms were not observed. Because of the sensitivity of the analytic procedures used and because control experiments were carried out involving D-allose and D-altrose, which would have appeared if transformations involving Carbon 3 had occurred, it was concluded that they did not occur, at least to any greater extent than about one percent of the starting material.

In the disaccharide system, the parallel transformation reactions between cellobiose, cellobiulose and 4-O-D-glucosylmannose were also observed. It appears that this is the first reported observation of these particular reactions.

In all of these transformation reactions, the reversible gluco-fructo aldo-keto isomerizations had rate constants orders larger than the manno-fructo isomerizations. Further, all the isomerization reactions had rate constants significantly larger than the reversible gluco-manno epimerization reactions. Thus, the kinetic data clearly show, and for the first time, that the gluco-manno epimerization reaction is energetically very unfavorable compared with the observed aldo-keto

isomerization reactions. Presumably, there is some higher energy barrier in the formation of possible intermediates and along possible pathways to and from the mannose molecule. Possibly (and if these transformation reactions at least begin while the molecule is in a cyclic structure), this is related to the postulated trans-diaxial conformation of the 1,2 hydroxyls of α -D-mannose as opposed to the trans-di-equatorial conformation of these hydroxyls in β -D-glucose. In this connection, it may be pertinent to recall the observation of a somewhat anomalous physiological reaction with mannose wherein it was noted that mannose was absorbed by rate at only about 12% of the rate of D-glucose and that for the absorbed mannose the glycogen deposition was much smaller than for D-glucose (75).

It is interesting to note in connection with the gluco-manno isomerization that the finding in this study of equal values for the rate constants in each direction of this reaction is in harmony with the early work of Wolfrom and Lewis (76) and Greene and Lewis (77) on the observation of an "equilibrium" isomerization between 2,3,4,6,tetra-O-methyl-D-glucose and 2,3,4,6,tetra-O-methyl-D-mannose. While they used quite different reaction conditions (1 molar carbohydrate in 0.018M calcium hydroxide and reaction run to exhaustion of the base, compared with the present 0.002 molar carbohydrate in 1M sodium hydroxide), they reported a 50/50 "equilibrium mixture" of the epimers. This finding is in harmony with the present finding of equal rate constants for each direction of this reversible reaction.

In this early work and subsequently throughout the literature there appear statements and allusions to the conclusion that the transformation reactions proceed to an equilibrium state. The composition of the "equilibrium mixture" has been given. It is now possible to conclude from this present kinetic study that there is no equilibrium state in the system in so far as transformation reactions are concerned. Providing base remains available, the transformation reactions will continue, and the concentrations of the several carbohydrates will decrease to

essentially zero as required by the associated degradation reactions. The system will undoubtedly reach an equilibrium state between the base and an array of degradation products but never between the base and the transformed carbohydrates.

A further conclusion with respect to the transformation reactions is that in the present system the mannose moieties which appear have more likely come from the glucose moiety via the fructose than directly from the glucose. This conclusion is supported by the D-glucose-1-d-studies of Topper and Stettin (78) made in 1951. When they reacted D-glucose-1-d in saturated lime water at 25°C., they found the D-mannose produced contained only 44% as much deuterium as the starting D-glucose, but the D-fructose produced contained the same amount of deuterium as the starting D-glucose. If the pathway between glucose and mannose involved only the removal and replacement of a proton at C2, then the mannose should have contained the same amount of deuterium as the glucose. However, the subsequent work of Sowden and Schaffer (79) in 1952 with glucose in a $D_2O-Ca(OD)_2$ system and the early work of Wolfrom and Lewis (76, 77) indicated the glucose-to-mannose pathway was a direct one only. It now appears that both the direct and the detour pathways are used with the detour being energetically preferable under the present reaction conditions.

Having now available the transformation reaction rate constants for both a monomer and the equivalent disaccharide system, it is possible to conclude that for the reaction conditions studied the rate constants of all equivalent transformation reactions in the two systems are essentially the same with the single, but markedly outstanding, exception of the disaccharide cellobiose to cellobiulose isomerization being about double that of the equivalent monomer glucose to fructose isomerization. As the transformation reactions probably involve only Carbons 1 and 2 in either the monomers or the disaccharides, the similarity of transformation

rate constants might be anticipated. The single exception though is interesting and may be related to the peeling reaction as discussed below.

The data obtained in this study clearly confirm the existence of the peeling reaction and quite possibly by the mechanism suggested in Fig. 2. However, it is noted that for the reaction conditions used it appears as shown in Fig. 9 that only about 80% of the starting cellobiose molecules proceed through cellobiulose (either directly or in very small amount via glucosylmannose), and on to the peeling reaction. About 10% of the cellobiose molecules are converted directly (GG to D in Fig. 6) to unknown products. A further 10% are converted on a non-peeling reaction route via cellobiulose (GF to H in Fig. 6). About 1-2% of the cellobiose molecules are degraded on a nonpeeling reaction route via glucosylmannose (GM to E).

In connection with the relatively higher rate constant for the cellobiose to cellobiulose isomerization, as noted above, the following speculative conclusion is offered. Assuming, as depicted in Fig. 1, that a 1,2-enediol is the intermediate from which the 2-keto molecule is formed from the aldose, then in the case of the biose isomerization, the next step of proton extraction preferentially occurs from the hydroxyl at Carbon 2 in the 1,2-enediol rather than of the carbon bound proton at Carbon 1 because the glucosyl radical substituted at Carbon 4 has a greater inductive effect than the equivalent hydrogen at Carbon 4 of the monomer, and thus the oxygen-bound proton at Carbon 2 of the biose is an easier leaving group under nucleophilic attack than the equivalent proton in the monomer. On this basis, it is predicted that for the equivalent rate constant its value would increase to a limiting value with an increase in the number of units in the $\beta(1\rightarrow4)$ glucan.

No precise quantitative data were obtained in this study with respect to the postulated stopping reaction. However, some semiquantitative chromatography data indicated the presence of a reaction product (appearing late on the chromatogram and thus suggestive of a molecule containing a carboxyl group) which on hydrolysis gave glucose. The amounts of glucose produced were in approximate accord with what might arise from the sum of the molecules D and H arising from cellobiose and cellobiulose, respectively. There was an indication that a higher percentage of the starting material appeared as glucose from the spent system when the starting material was cellobiulose. This indication agrees with the calculated findings and suggests that the route to the stopped molecule, glucosylmetasaccharinic acid, is preferentially via cellobiulose. On the basis that molecules D and H and possibly also E are largely stopped biose molecules, then perhaps as much as 10-15% of a given amount of cellobiose proceeds under the conditions used to the glucosylmetasaccharinic acid molecule as hypothesized for the stopping reaction. This tentative conclusion is in fair agreement with the recent studies of Lindberg, Theander, and Uddegard (80) who report a 3% stopping reaction for cellobiose in 0.02 molar sodium hydroxide at 90°C. and an increase to 4.5% at 60°C.

There are some other matters arising out of this study which are considered to warrant conclusions.

In the rather extended study of elution gradient variation carried out in this research in seeking a gradient to resolve the cellobiulose-glucosylmannose and fructose-mannose pairs, it was quite evident that changes in the concentration and pH gradients of the eluent caused pronounced shifting in elution times among these molecules. This suggests and leads to the conclusion that well-designed experiments on the chromatography of borate complexed carbohydrates could yield useful information on the conformations of the carbohydrates. Control molecules could be deoxy or substituted derivatives.

Having obtained a set of rate constants for the reaction system arising from cellobiose as shown in Fig. 6, it is concluded that the concentrations of the six carbohydrates can be calculated on the computer, and the related curves plotted, for any reaction time for any system arising from any starting mixture of the six when the present reaction conditions are used. For example, such a plot is given in Fig. 14 for a mixture starting with equimolar concentrations of each of the six. It will be interesting to test this plot experimentally. This predictive application of these types of data suggests its potential value in automated control in industrial processes.

As a further and final conclusion from this work, it now appears possible to proceed into a rather comprehensive study of these transformation and degradation reactions. From either a theoretical, physiological, or industrial point of view a program of research involving variation of such parameters as temperature, base concentration, cation type, additional inorganic anions, enzymes, and the structure of the starting molecule should yield kinetic data which could be a penetrating insight to these interesting and important reactions. Some tools we now have; it remains but to do the job.

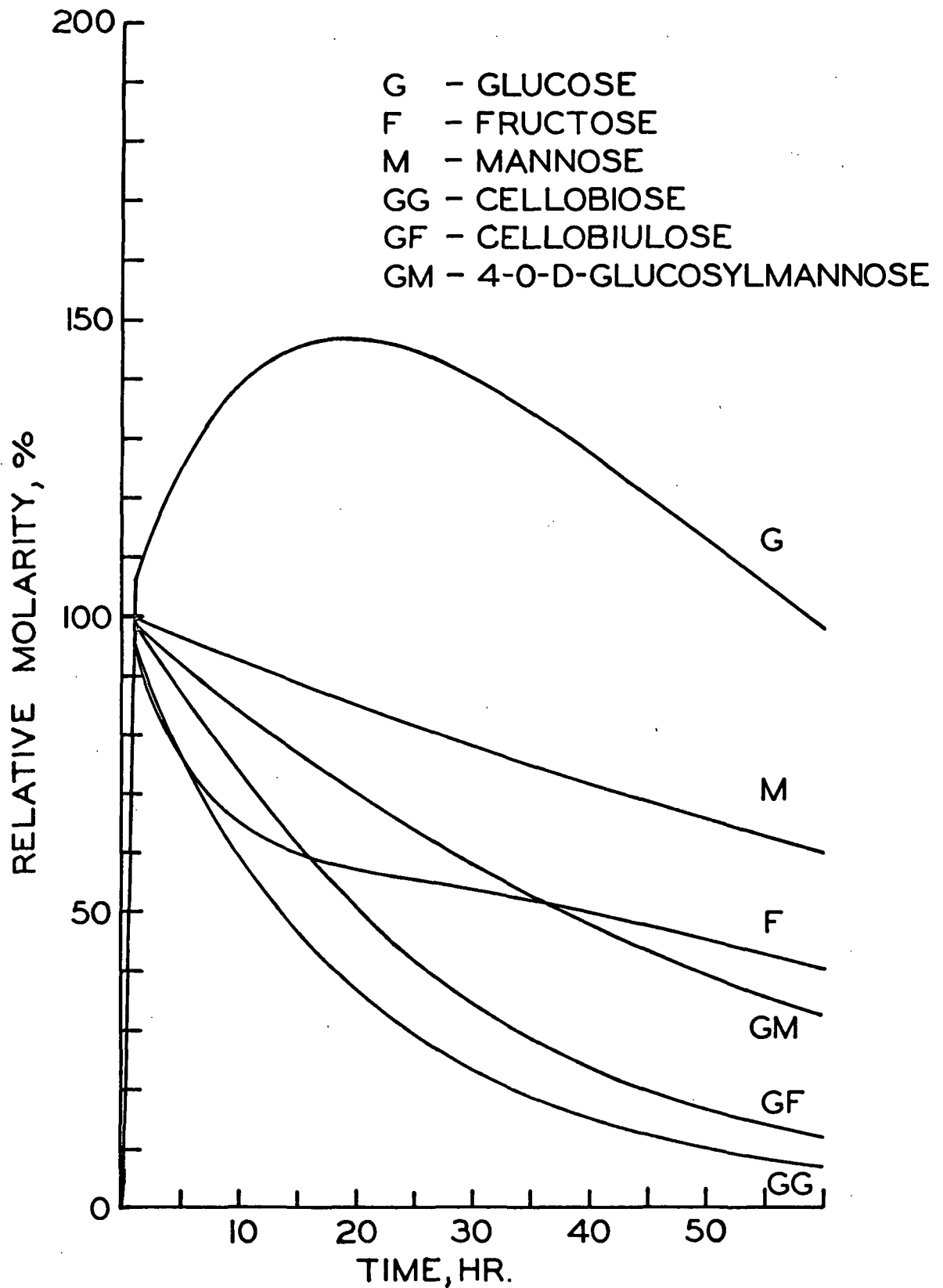


Figure 14. Calculated Plots for Equal Initial Molarities of Glucose, Fructose, Mannose, Cellobiose, Cellobiulose and 4-O-D-Glucosylmannose in 1M Sodium Hydroxide at 22°C.

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APPENDIX I

MATERIALS AND SOLUTIONS

CARBOHYDRATES

The glucose, fructose, mannose, and cellobiose used were from commercial sources. The cellobiulose and 4-O-D-glucosyl-D-mannose used were synthesized specifically for this study. Following are the details of the preparation and synthesis of all these carbohydrates as used in this research.

Each of the carbohydrates was assessed for chromatographic purity and then further purified as necessary and possible. A chromatographically pure material was taken as one which gave a single symmetrical peak when assayed by the chromatographic procedures to be used in subsequent analytical work and by those procedures but with at least one different column elution gradient. The criterion that purity be established by at least two different gradients was imposed to minimize overlooking the unlikely event that more than one material resulted in a single peak for a particular gradient. A carbohydrate producing a minor peak (less than 1%) at a point on the chromatogram not occupied by any of the other carbohydrates of interest (or a very minor peak of a carbohydrate of interest) while not chromatographically pure was accepted in some cases. In assessing the purity, an aqueous solution (boiled, N₂-saturated, distilled water) of the carbohydrate to be chromatographed was prepared so that it was 0.0100-0.01500 ± 0.00005 g./100.0 ml. in the carbohydrate, 0.33N in NaCl, and 0.12M in H₃BO₃ at pH 7-8. Thus, this solution contained essentially the same concentrations of the several inorganic ions present in a reaction run sample as prepared for chromatography. While any interrelationships which may exist between inorganic ions and their concentrations in the column sample and chromatography results are not fully known, there was evidence that even though the column sample was

a relatively small volume (0.1-1.0 ml.), a given quantity of a carbohydrate gave different chromatographic results with different sample solution compositions. A volume of this solution containing 500-1500 µg. of the carbohydrate was then chromatographed. By these procedures, chromatographic purity can be assessed to within $\pm 0.1\%$. In the case of a carefully crystallized carbohydrate, this chromatographic purity is probably usable as absolute purity.

D-Glucose:

Baker AR Dextrose, anhydrous powder, Lot 25814. Found to be $\dagger 99.9\%$ pure and dry as received. Used as received.

D-Fructose:

Pfanstiehl C.P. special, Lot 3155. Found to be $\dagger 99.9\%$ pure. Used after drying over Drierite.

D-Mannose:

Matheson, Coleman and Bell, and Pfanstiehl, Lot 6799-A. Both found to be about 98% pure as received. MCB material recrystallized from methanol-isopropanol (81), dried over Drierite, and used.

Cellobiose:

Pfanstiehl, Lot 7236. Found to contain about 1% glucose. This material was not improved by slow recrystallization (65 g. from 150 g.) from methanol-water. A further slow recrystallization from acetone/water, 2/3, v/v (25 g. from 56 g.) gave a material containing about 0.15% glucose and no other impurity. This product was dried over Drierite and used.

Cellobiulose:

This compound, 4-O-D-glucosyl-D-fructose (GF), was prepared from cellobiose (GG) by a route based on that employed by Corbett and Kenner (21). The

method utilizes the conversion of the aldose to the isomeric 2-ketose as occurs in an alkaline solution of the aldose. Details of the preparation procedures are given below.

4-O- β -D-Glucosyl-D-Mannose:

The synthesis of this material was based on the 1926 procedure of Brauns (55). In this, dry cellobiose octaacetate was reacted with anhydrous hydrogen fluoride to yield hexa-O-acetyl-4-O- β -D-glucosyl-D-mannosyl fluoride. This material was acetylated to give octa-O-acetyl-4-O- β -D-glucosyl-D-mannose which was deacetylated to give the desired compound, 4-O- β -D-glucosyl-D-mannose. The details of the preparation procedures are given following those for cellobiulose.

PREPARATION OF CELLOBIULOSE--ORIENTING EXPERIMENTS

The material used for kinetic runs in this study (GF-1) was prepared through the following preliminary small-scale studies and subsequent larger quantity runs.

Thirty-four semimicroscale runs with various bases (see Table II) and times were made to guide a choice of conditions in seeking to improve the yield of cellobiulose from cellobiose.

In these, 0.25 ml. of 7% aqueous cellobiose solution and 3 ml. of the base solution (about 0.04M) was placed in a 110 by 15-mm. test tube. The array of rubber-stoppered tubes was arranged in a wire rack and set in the water bath at 30°C. At desired intervals, tubes were withdrawn and 0.5 ml. of IR 120-H resin and 1 drop of phenolphthalein indicator added and the mixture well shaken. This system turned acid in 3 to 4 minutes. Two (exactly) milliliters of clear supernatant were withdrawn and reduced to dryness in a vacuum oven or desiccator. As the calcium systems contained fine solid material in suspension, they were

TABLE II

SUMMARY OF REACTION OF APPROXIMATELY 0.014M CELLOBIOSE IN VARIOUS AQUEOUS BASIC SOLUTIONS AT 30°C.

Run	Base	Approx. Base Strength	Time, hr.										
			0.5	1	2	2.5	3	5	6	17	18	47.5	91.5
9	Ba(OH) ₂	0.04M	5					30	40	46		80	85
							15	20	15		10	5	
10	NaOH	0.04M					35	40	50		85	90	
							10	15	10		5	3	
11	Ca(OH) ₂	0.04M					40	70	90		99	100	
							12	10	3		0	0	
12	Ba(OH) ₂	0.04M	5	10	15	20							
			2	5	10	12							
13	NH ₄ OH	9.4M	0	5			10	30				40	
			0	0			2	5				10	
14	Pyridine	30M	0	0			0	0				0	
			0	0			0	0				0	
15	Piperidine	1M	5	10			25	35				50	
			2	5			11	12				8	

Note: Upper number = approx. percentage cellobiose reacted. Lower number = approx. percentage of original cellobiose present as cellobiulose.

The molarities for Runs 13, 14, and 15 were based on K_0 's of 1.82×10^{-5} , 1.71×10^{-9} , and 1.6×10^{-3} , respectively, and thus were an attempt to approach 0.04M hydroxyl in those solutions.

filtered before the resin was added. Water (0.25 ml.) and 2 drops of methanol were added to each dry residue and the resulting solutions stored in the cold room in capped 1.5-ml. plastic vials, pending chromatographic examination. Spots from these solutions were developed in the solvent 9:2:2, v/v, ethyl acetate:acetic acid: water on Whatman No. 1 paper and the sheets dipped in silver nitrate, etc. The results from these runs are summarized in Table II. From these experiments it was concluded that an improved yield of cellobiulose from cellobiose could be obtained in 0.04M barium hydroxide at 30°C. with about 5 hours' reaction time. The following additional observations were made on the basis of these experiments.

(a) No reaction products other than cellobiulose, glucose, and fructose appeared on the chromatograms from the piperidine system, even when the cellobiose was some 50% reacted. This would appear to be an interesting situation for further study.

(b) Ammonium hydroxide appeared to give significantly fewer nonsugar reaction products than the other bases used except piperidine.

(c) The colors of the reaction mixtures and the chromatograms at various times strongly suggested that the alkaline degradation of cellobiose is quite sensitive to the specific cation involved.

Based on these semimicro runs, a larger amount of cellobiulose was produced in a series of three runs at 30°C. in 0.04M barium hydroxide for 5 hours. The cellobiose reclaimed from the first run was used for the second run, and that reclaimed from the second for the third.

Twenty grams of cellobiose were dissolved in 200 ml. of freshly boiled distilled water in a 500-ml. flask. After the flask and contents had stood one hour in the water bath at 30°C., 1.26 g. of barium hydroxide (octa hydrate) were added.

After 5 hours the reaction was quenched with IR 120-H resin and the combined filtrate and washings reduced from 500 to 35 ml. giving a very light-colored, heavy sirup. Within 15 minutes, crystals began to form in this system, and after about 5 hours, a heavy crop of crystals had come down. Then 25 ml. of methanol were added on the supposition that cellobiulose was more soluble than cellobiose in an aqueous methanol system containing both. Chromatographic examination of the crystalline material showed it was over 99% cellobiose and free of cellobiulose. The crystalline cellobiose was filtered off and washed with methanol and a second run for cellobiulose made as above, using this reclaimed cellobiose as starting material. The filtrate and washings were reduced to 15 ml. and a second crop of crystals recovered, and then a third. Table III below summarizes the progress of this separation of cellobiose from cellobiulose by fractionation precipitation in aqueous methanol, as determined by chromatographic monitoring.

TABLE III

COMPOSITION OF SUCCESSIVE CRYSTALLINE CROPS (X) AND
REMAINING SUPERNATES (S) IN THE FRACTIONATION PRECIPITATION
FROM AN AQUEOUS METHANOL SOLUTION CONTAINING
CELLOBIOSE, CELLOBIULOSE, AND GLUCOSE

	Approximate Molar Ratios		
	Cellobiose	Cellobiulose	Glucose
Starting Solution	80	15	5
X1	99.5	--	0.5
X2	99.7	0.1	0.2
X3	98	11	1
S1	55	30	15
S2	35	45	20
S3	15	80	5

A series of experiments was made to examine the possibility that cellobiulose could be preferentially crystallized from aqueous ethanol solutions of cellobiulose, cellobiose, and glucose. No useful concentrating of the cellobiulose was achieved.

The aldose materials (cellobiose and glucose) in the combined S3 supernates from these cellobiulose runs were then oxidized with hypiodite using procedures based on the method of Kline and Acree (83, 84) to convert the aldoses to carboxylic acids.

The various aqueous methanol cellobiulose systems were combined, reduced to dryness, diluted with water, and again reduced to dryness and then made up to 500 ml. with water. This solution contained about 1% cellobiulose. To 10.0 ml. of this solution were added 2 ml. of 0.1M iodine, then dropwise 3 ml. of 0.1M sodium hydroxide as the system was well stirred with a magnetic bar stirrer. These alternate additions continued until a total of 11 ml. of 0.1M iodine and 16.5 ml. of 0.1M sodium hydroxide had been added. This took 15 minutes. After standing 2 minutes, the system was brought to pH 2 with 0.1M hydrochloric acid, and the unreduced iodine determined with 0.1M sodium thiosulfate. It was found that 4.3 ml. of 0.1M iodine had been reduced. On this basis there was added a total of 220 ml. of 0.1M iodine and 330 ml. of 0.1M sodium hydroxide to the cellobiulose system in 20 and 30-ml. portions as before. This took 70 minutes. After standing 5 minutes, the system was found to contain unreduced iodine, and thus it was concluded that sufficient iodine had been added to oxidize the aldoses. The system was stirred for 5 minutes with 200-ml. IR 120-H to remove metallic cations. The resin was then filtered off and washed. To the well-stirred system were added 20 grams of silver carbonate. A lemon-yellow precipitate (silver iodide) began to form almost immediately. The system was allowed to stand overnight, with the stirring continued, and during this time it separated into a dark sludge and a clear supernate which

gave a positive halogen test. The sludge was then filtered off, an additional 20 grams of silver carbonate (finely ground) were added, and the system was stirred for 15 minutes. At this time no positive test for halogen was obtained in the filtered liquor. Resin IR 120-H was then stirred in until no positive test for silver resulted (this required 120 ml.), and the system (whose volume was now 2500 ml.) was filtered, again giving negative tests for silver and halogen. The volume was then reduced to 1000 ml. (pH now 3.2), and the test for halogen was negative, with a possible trace of silver. To the well-stirred system was added solid calcium carbonate to pH 6.5, and then saturated calcium hydroxide solution to pH 7.3 when a fine precipitate began to form. The system was reduced to 400 ml., and a muddy-colored precipitate filtered off. The volume was then reduced to 200 ml., when a few black specks (possibly silver) appeared. Forty ml. of IR 120-H resin were then stirred in, and the system was checked and rechecked for silver in the supernate; all of these tests were negative. The resin was then filtered off and the volume reduced to 100 ml. Chromatographic examination showed this system to contain no cellobiose, 2% cellobiulose, and traces of glucose and other materials which were well separated from cellobiulose when developed by the solvent 8:2:1, (v/v ethyl acetate:pyridine:water). The volume was then further reduced to 20 ml., and cellobiulose separated from this concentrate by paper partition chromatography in the following manner.

Forty sheets of Whatman No. 3MM paper, 9-1/2 by 12 in., grain long, were prepared. A 0.25-ml. streak of the cellobiulose concentrate was applied to each side of these forty sheets across the full width of the sheet at the starting line. These sheets were run in the 8:2:1 solvent. The progress of the cellobiulose fraction was followed by monitoring with the peel-strip technique developed by MacLaurin, Crossman, and Green (85). Some 45 hours' development moved the cellobiulose about 9 inches, which was suitable for excision and elution. Two 2-in. wide bands were

cut from each sheet bracketing the cellobiulose position. Each six of these strips were assembled into a pile with interleaf strips of Whatman No. 1 of the same width but twice the length. The heavy paper strips were centralized on the longer strips and the pile stapled together above and below the heavy strips. The pile was wrapped in 4-mil. polyethylene film extending half an inch beyond each end of the heavy paper strips. Rubber bands were placed around the pile over the polyethylene at 2-inch intervals. One end of the group of projecting strips of light paper was trimmed to a 60° point and the other end clamped with stainless steel chromatography clamps, between a pair of 2-1/2 by 2-1/2-inch. pieces of glass slide in such a way that the polyethylene film wrapper extended about 1/2 inch into the bite between the slides. A 2 by 4 by 18-inch enamel pan served as an elution trough. The prepared bundles were hung over the side of the trough and the eluent directed through a funnel into a 1000-ml. RB flask. Some bundles were eluted with methanol in the hope that a water-free system might more easily yield a crystalline cellobiulose product. Others were eluted with water. About 150 ml. were eluted from each bundle. Chromatographic examination of the eluted strips showed no cellobiulose not eluted. All elutions took place underneath a polyethylene tent. Chromatographic assays of these eluents showed that they contained essentially only cellobiulose. Various lots of the cellobiulose eluent were manipulated over a period of some three months in unsuccessful efforts to obtain crystalline material. All cellobiulose systems were subsequently converted to aqueous systems. These were combined, clarified with Darco G carbon, reduced to almost dryness in a vacuum oven at 45°C./29 in., and then stored over Drierite in a vacuum desiccator at 28 inches vacuum. About 3 ml. of a thick, clear, honey-colored sirup were obtained. Chromatographic analysis of this material and its hydrolyzate showed it to be essentially pure cellobiulose. The hydrolyzate (1M sulfuric acid for 6 hours at 100°C.) showed

glucose and fructose only and in about equal amounts. This material (GF-64)* was dissolved in 100 ml. methanol and stored in a refrigerator at 40°F.

PREPARATION OF CELLOBIULOSE--FOR KINETIC RUNS

Based on this orienting experience, two reaction runs (RR 29-30) were made for the production of cellobiulose for kinetic runs, using the following conditions and steps:

1. Reacted 50 g. GG in 500 ml. 0.015M $\text{Ba}(\text{OH})_2$ for 6.5 hr. at 30°C.
2. Reaction quenched with about 200 ml. Amberlite resin IR 120-H.
3. Resin filtered off and washed. Filtrate and washings (about 1000 ml.) combined and reduced to dryness at 40°C. and 29 inches vacuum. Added about 250 ml. MeOH which resulted in a supernate (\underline{S}_1) and a ppt. (\underline{X}_1).
4. Filtered off \underline{X}_1 ; yield 40-45 g., about 99% GG.
5. Concentrated \underline{S}_1 + \underline{X}_1 washings at 40°C. and 29 inches vacuum to about 75 ml. $\rightarrow \underline{S}_2$ + \underline{X}_2 .
6. Filtered off \underline{X}_2 ; yield 1-2 g., about 97% GG.
7. Concentrated \underline{S}_2 + \underline{X}_2 washings at 40°C. and 29 inches vacuum to dryness; added about 75 ml. MeOH $\rightarrow \underline{S}_3$ + \underline{X}_3 .
8. Filtered off \underline{X}_3 ; yield 0.25-0.5 g., about 90% GG.
9. \underline{X}_1 's + \underline{X}_2 's reused as starting GG for following runs. \underline{X}_3 's discarded.
10. \underline{S}_3 's combined \rightarrow dryness at 40°C./29 in. vacuum + 500 ml. boiled, distilled H_2O . By chromatography found to be about 1% GF, 0.2% GG, and 0.2% G.

* $[\alpha]_D^{20} = -75 \pm 2^\circ$ (c = 10 [anhyd. basis] H_2O) from sirup at estimated 10% H_2O .
Reported (21): $[\alpha]_D^{23} = -60.1^\circ$ (c = 2.40, H_2O).

11. Aldoses in combined S_3 's oxidized by hypiodite according to the method of Kline and Acree (83, 84), and the resultant solution deionized by use of Amberlite IR 120-H to pH 1.7--filter, wash = 1500 ml. → 1000 ml. (40°C./29 in. vacuum) = pH 1.6 + Amberlite IRA 400-OH--pH 9--filter off resin + IR 120-H slowly → pH 6.8, filter off resin → crystal clear, colorless filtrate 1500 ml., pH 7.5 → 600 ml. (40°C./29 in. vacuum) = GF-1 system.
12. Paper chromatography showed this system still contained a trace of G, so Step 11 above was repeated on an appropriately reduced scale, and then no chromatographic evidence of other than GF.
13. GF-1 very light honey-colored, very viscous sirup (5 ml.) at 40°C./29 in. vacuum.
14. GF-1 sirup + 40 ml. MeOH = light green-yellow solution, heated on water bath with swirling to near boiling (about 75°C.) → dusty ppt. beginning to form, cooled + 100 ml. MeOH + 200 ml. Et-O-Et → fairly copious white floc with light green-yellow supernate.
15. The GF-1 white floc was filtered off, washed with acetone and ether, all under dry N_2 . The yield was about 5%. This GF-1 solid was amorphous, pure white, and very hygroscopic. It was weighed up under dry N_2 when kinetic run solutions were prepared. The remainder was stored under ether.

PREPARATION OF 4-O-β-D-GLUCOSYL-D-MANNOSE

The procedures of Brauns (55) were modified by using commercially available anhydrous hydrogen fluoride directly from a cylinder in place of generating it from potassium acid fluoride. A polypropylene reaction vessel was used in place of vessels of copper and gold. In the acetylation step, perchloric acid served as catalyst in place of fused zinc chloride and in the final deacetylation, sodium

methoxide replaced barium methoxide as catalyst. Cations were removed with resins rather than by precipitation as insoluble salts. Final crystallization of the product was from stirred, boiling 95% ethanol in place of cold absolute ethanol. These modifications resulted in the anhydrous form of the final product at 23% of theoretical yield compared with the reported about 8% yield as the monohydrate. Details of the preparation are as follows. Gloves, eye protection, apron, and safety shield were used whenever hydrogen fluoride systems were being handled.

A 1000-ml. heavy-walled polypropylene screw-cap bottle (Matheson Scientific No. 4336-40) containing 70 g. of dry cellobiose octaacetate* was cooled in a thermos bath of acetone-carbon dioxide dry ice. By means of a 1/4-inch polyethylene tube, 140 g. of anhydrous hydrogen fluoride gas was slowly (3-1/2 hr.) bled from a cylinder container and condensed in the bottom of the cooled bottle onto the carbohydrate material which went into solution during the first two hours. The bottle and contents were weighed from time to time to assess the progress of transfer of the hydrogen fluoride. When sufficient was transferred, the bottle cap was tightly screwed on and the capped bottle set aside under the hood at room temperature for 6-1/2 hours. The bottle and contents were then re-cooled in the acetone-carbon dioxide ice bath. The bottle was opened and 300 ml. of similarly cooled chloroform cautiously added to the reaction mixture.

The mixture was then dumped and rinsed (100 ml. cooled chloroform) into a polyethylene vessel containing 800 ml. distilled water, 800 ml. crushed ice from distilled water, and 400 ml. chloroform. This mixture was stirred vigorously with a polyethylene rod. After the ice had melted and the water layer appeared

*This material was the generous gift of Dr. L. S. Schroeder, by whom it had been prepared in the usual way by acetolysis of cellulose.

free of entrained solid, the water layer was removed by decanting. The chloroform layer was repeatedly stirred with fresh distilled water (about 20 liters) followed by decanting until the water layer was essentially neutral to Congo Red paper. Finally, about 25 g. of sodium bicarbonate were added to the water layer to give pH 8. The chloroform layer was now drawn off in a separatory funnel, combined with 300 g. of anhydrous sodium sulfate and set aside to dry for 12 hours. The sodium sulfate was filtered off and washed with chloroform. The filtrate and washings (1500 ml.), clear, green-gold color, were concentrated at 30°C./25 in. vacuum to about 30 ml. of heavy sirup.

To this sirup was added 470 ml. of acetic anhydride. A clear, green-gold colored solution formed within 5 minutes. To this solution was added 3 ml. of 72% perchloric acid. The color of the system immediately darkened to mahogany red. The reaction flask was placed in a water bath at 35-40°C. for one hour and given an occasional swirling shake. The reaction system was then dumped and rinsed (50 ml. acetic anhydride) into a 4-liter beaker containing 1 liter of crushed ice from distilled water and 1 liter of distilled water. The system was vigorously stirred for the first 15 minutes, then occasionally for the next 2-1/2 hours. A brownish, lumpy solid (50 ml.) was then filtered off with suction and washed with 20 liters of distilled water.

This solid was dissolved in a boiling mixture of 1 liter of 95% ethanol and 1 liter of absolute ethanol to give a dark brown solution. This solution was set aside to cool with stirring on a magnetic stirrer. After 15 minutes, long fibrous crystals began to fall out. After 3 hours, the system was a fairly heavy slush of fibrous crystals. These were filtered off under suction through Whatman's glass filter paper (GF/A) and washed on the filter with 95% ethanol. No additional product could be recovered from the mother liquor. The combined filter cakes were redispersed in 2-1/2 liters of distilled water. The product was filtered off again

and washed on the filter with distilled water. The wet crystals were dried at 43°C./29 in. vacuum to constant weight (24 g.) in 12 hr. This material was a snow-white, asbestoslike crystalline product and had a corrected m.p. of 204°C. compared with literature values of 202-3°C. for glucosylmannose octaacetate and 229°C. for cellobiose octaacetate.

This product was then deacetylated. The 24 g. of dry product were suspended in 250 ml. of dried and redistilled methanol. While the suspension was rapidly stirring, 30 ml. of sodium methoxide solution (0.5 g. sodium in 100 ml. dried and redistilled methanol) were added. In 15 minutes, the suspension became a clear, brownish solution. After 45 minutes, 10 ml. of IR 120-H resin were stirred in, and the pH went from 8 to 6. The resin was filtered off and the solution concentrated to 15 ml. of very heavy, honey-colored sirup at 35°C./28 in. vacuum. This sirup was brought into solution in a boiling mixture of 100 ml. absolute ethanol and 200 ml. 95% ethanol. The light-colored solution was set aside to cool while stirring slowly on the magnetic stirrer. Overnight a good crop of fine-grain crystals developed. These were filtered off and washed with 95% ethanol and then with acetone and sucked dry. Chromatographic examination of these crystals showed them to be better than 99% glucosyl mannose. No further product could be recovered from the mother liquor.

The light-colored crystals (8.7 g.), m.p. 177°C., were redissolved in 150 ml. H₂O, and a small amount of floating scum separated off by filtration. To the filtrate was added 5 cc. of a 50/50, v/v mix of Darco-G 60/Celite 545, and after 5 minutes' stirring the solids were filtered off and washed with water through a three-sheet layer of Whatman's GF/A glass filter paper to give a very clear, carbon-free, colorless solution. This was concentrated to 10 ml. of water-clear, very heavy sirup at 35°C./29.5 in. vacuum. The sirup was taken up in 100 ml. 95% ethanol; then a further 75 ml. 95% ethanol were added and the system heated to near

boiling to give a clear, colorless solution. This solution was set aside to cool, stirring slowly. Crystals began to form immediately. After 12 hours, the crystals were filtered off and washed with 95% ethanol, then with acetone, and sucked dry.

The crystalline product (8.3 g. dry) was snow white and had a corrected melting point of 178.5-179.5°C. After drying a sample of the product 4-1/2 hr. at 100°C./26 in. vacuum to constant weight, the loss in weight was 5.6% of the undried material. The melting point of the dried material was unchanged at 178.5-179.5°C. Reported 174-175°C. (55) and 175-176°C. (54, 86), all presumably uncorrected values. Optical rotation, based on dry anhydrous material, was found to be: initial, $[\alpha]_D^{20} = +6.3$ ($c = 2.2$, H₂O) and an equilibrium value, after 60 minutes, of $[\alpha]_D^{20} = +6.5$. Reported (56) initial, $[\alpha]_D^{20} = +14.46^\circ$ ($c = 5.2$, H₂O) and an equilibrium value, after 75 minutes, of $[\alpha]_D^{20} = +6.2^\circ$. The material had a chromatographic purity of 99.8% with the 0.2% being cellobiose. Its chromatographic behavior was identical with a sample of 4-O-β-D-glucosyl-D-mannose which was a gift from Dr. N. S. Thompson (87). Thus, on the basis of method of preparation, melting point, chromatographic behavior, its transformation to cellobiose and cellobiulose in alkaline solution, this material was 4-O-β-D-glucosyl-D-mannose.

SOLUTIONS

0.12M H₃BO₃, pH 8.50

30.00 ± 0.01 g. H₃BO₃ (Mallinckrodt AR) + 4000 ± 1 ml. (72°F.)
distilled water + nominal 12M KOH to pH 8.50 ± 0.02 at 72°F. (about 10 ml. KOH solution required). Solution filtered through a Whatman GF/A, 5.5-cm. glass filter paper supported on a Millipore sintered disk-and-funnel unit. Stored in clear glass bottle at 72°F.

0.15M $K_2B_4O_7$, pH 9.4

184 ± 0.05 g. $K_2B_4O_7 \cdot 4H_2O$ (Mallinckrodt AR) + 4000 ± 1 ml. (72°F.)

distilled water and filtered as above. Stored in clear glass bottle at 72°F.

0.50M $K_2B_4O_7$, pH 9.7

611.0 ± 0.5 g. $K_2B_4O_7 \cdot 4H_2O$ (Mallinckrodt AR) + 4000 ± 1 ml. (72°F.)

distilled water and filtered as above. Stored in clear glass bottle at 72°F.

Orcinol/70% H_2SO_4

95.5-96.5% sp.gr. 1.84 H_2SO_4 , slowly added with cooling to 600.0 ± 1 ml. (72°F.) distilled water in a 2-liter volumetric flask. The addition of the final 1-2 ml. of H_2SO_4 to the mark is done after the well-mixed H_2SO_4/H_2O solution has stood at least 12 hours at 72°F. The 2 liters of 70% H_2SO_4 is slowly poured onto $3,500 \pm 0.001$ g. of near-white color orcinol monohydrate (K and K Laboratories) in a 2-1/2 liter wide-mouth brown glass bottle. The orcinol/ H_2SO_4 solution is stirred with a magnetic stirrer for about 1/2 hour and then carefully examined, particularly at the scum line, for complete solution of the orcinol. The color of this solution deepens with time, and in this research a solution more than 24 hours old was not used for quantitative work. It appears, though, that orcinol solutions even a week old are useful for qualitative work.

1M NaOH

In a Teflon beaker, 500 g. NaOH (Mallinckrodt AR) dissolved in 500 ml. boiled, N_2 -saturated, distilled water. This "lye-oil" let stand under N_2 in a thick-walled, 1-liter, tightly capped polypropylene bottle at room temperature for no less than two weeks to allow Na_2CO_3 to settle out. Aliquots of the clear supernate were transferred by pipet to 2 liters of boiled, distilled, N_2 -saturated water in a polyethylene bottle in which the stock 1M NaOH

was stored. The 1M NaOH solution was standardized against $\text{KHC}_8\text{H}_4\text{O}_4$ (Mallinckrodt Primary Standard) to the phenolphthalein end point. The carbonate content of the 1M NaOH was determined by the carbon dioxide evolution method (TAPPI T 624 m-60) and found to be 0.002-0.003 molar. This carbonate content approaches that obtained by the most exacting technique of Allen and Low (88) for the preparation of "carbonate-free" sodium hydroxide solutions.

APPENDIX II

APPARATUS AND EXPERIMENTAL PROCEDURES

CONSTANT-TEMPERATURE BATH

A 40-gallon glass tank fitted with a constant-level overflow was fed cold water at a constant rate through an overflow constant-head device. Vigorous circulation of the water in the tank was maintained by a model A-5 Easy pump (2 gal./min.) in one corner and a submerged TEEL (IP598, 3 gal./min.) in the opposite corner. Heat input was through four 500-watt knife heaters, two of which were controlled by a Precision electronic relay (no. 62690) actuated by a 0-200°C. JUMO adjustable contact thermoregulator. Bath temperature was measured by a Cenco 9-200°C. no. 19245-4 thermometer which was calibrated to 4-inch immersion against Wesco thermometer 3406149, as calibrated by NBS test no. 165587 of March 17, 1961. This assembly maintained temperature with a precision of $\pm 0.05^\circ\text{C}$. and an accuracy of $\pm 0.05^\circ\text{C}$. (on the basis of the NBS calibration) for several months of continuous trouble-free operation.

RESIN COLUMN CHROMATOGRAPHY AND AUTOMATED ANALYTICAL EQUIPMENT

A schematic diagram showing the equipment and its arrangement as used for the chromatographic separation and quantitative analysis of the compounds of interest in reaction mixture samples is shown in Fig. 15. All components and accessories except the Haake Type FJ constant-temperature circulator were supplied by the Technicon Corporation, Research Park, Ardsley, New York 10502.

REACTION RUN PROCEDURES

A separate small reaction batch was run for any given reaction time. This approach provided better control and greater flexibility in getting data and

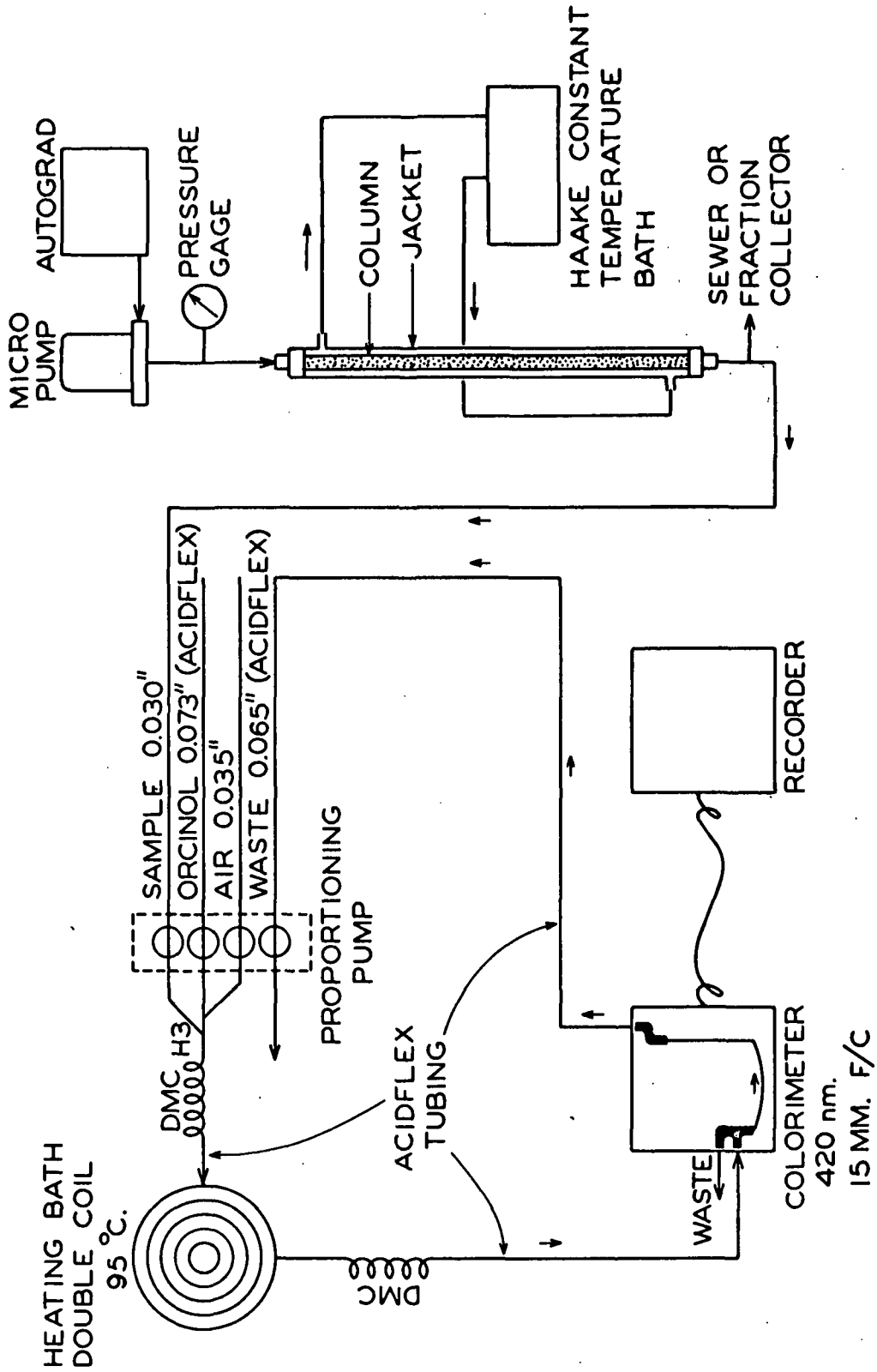


Figure 15. Apparatus - Schematic Diagram

repeat data at any desired reaction time than a scheme of drawing aliquots at intervals from one system. The procedures used were:

A 1.000 ± 0.001 -ml. volume of an aqueous solution (nitrogen-saturated, boiled, distilled water) of the chosen starting carbohydrate (1.000 - 1.010 ± 0.00005 g./ 100.0 ml.) was delivered under nitrogen by micropipet to a 200 -mm. x 25 -mm. pyrex tissue-culture tube which had a Teflon-lined black plastic screw top. Onto this carbohydrate solution was then delivered by pipet under nitrogen 25.00 ± 0.01 ml. of the sodium hydroxide solution. The tube cap was screwed on snugly and the system mixed by shaking. The tube was completely wrapped in three layers of aluminum foil and numbered with a felt pen. The wrapped tube was then placed in a wire test tube rack in the water bath. The rack was suspended on wires and under the influence of the vigorously swirling waters in the bath, it rocked to and fro and thus provided a gentle shaking motion to the reaction tube. The time at which the first sodium hydroxide hit the carbohydrate solution was taken as the zero reaction time. The reaction was quenched and a sample prepared for column chromatography as described in the next section.

REACTION RUN CHROMATOGRAPHY SAMPLE PREPARATION

A 25.00 ± 0.02 -ml. (72°F.) volume of about 1M HCl of strength just sufficient to result in a mixture with pH 1-2 was delivered by pipet to the 26.0 -ml. reaction system. The stopping time of the reaction was defined as when the last of the HCl from the pipet entered the reaction system. The mixture was shaken and then cooled in the constant-temperature bath. A 20.00 ± 0.02 -ml. (72°F.) aliquot of this acidic mixture was combined in a capped 1-oz. glass bottle with 10.00 ± 0.01 ml. (72°F.) of 0.36M H_3BO_3 , pH 9.0. This gave a solution, pH 7-8, 0.12M in H_3BO_3 and 0.33M in NaCl. The H_3BO_3 concentration was thus the same as at the beginning of the subsequent elution.

CHROMATOGRAPHY AND AUTO-ANALYZER CONDITIONS

The following conditions were established for column chromatography and Auto-Analyzer operation.

COLUMN

Six mm. i.d. x 750 mm. water-jacketed glass tube column.

Resin bed length of 700 mm.

RESIN

Technicon 3/28/VI. Spherical beads. Diameters: 11% > 30 μm .,
5% < 10 μm ., Av. diam. = 21 μm .

COLUMN TEMPERATURE

30.0 \pm 0.1°C., measured at the jacket outlet.

ELUENT FLOW RATE

1.00 \pm 0.01 ml./min. This gave a maximum pressure drop of about
500 p.s.i.g. across the column, which occurred during column washing
with 0.5M $\text{K}_2\text{B}_4\text{O}_7$.

ELUTION SYSTEM

In the nine-chambered, rectangular form Autograd:

Chambers 1-4, each 100 ml. of 0.12M H_3BO_3 , pH 8.50

Chambers 5-8, each 100 ml. of 0.15M $\text{K}_2\text{B}_4\text{O}_7$, pH 9.30

Chamber 9, empty.

PROPORTIONING PUMP TUBING

Sample from column	color code--black, Tygon, 0.030 in. i.d.
Air supply	color code--orange, Tygon, 0.035 in. i.d.
Orcinol/H ₂ SO ₄ reagent	color code--green, Acidflex, 0.073 in. i.d.
From colorimeter cell	color code--blue, Acidflex, 0.065 in. i.d.

MICROPIPET

Gilmont Micrometer Syringe: 2.0 ml. capacity, Cat. No. 7844,
Cole-Parmer, Chicago, Ill.

CHROMATOGRAPHY RUN PROCEDURES

The column top fitting was unclamped with the micropump off. The micropump was then started on 0.12M H₃BO₃, pH 8.50, and the top fitting slowly ejected and then lifted from the column just as excess liquid was about to overflow at the top of the column. The top fitting was then inverted and all air bubbles cleared from it by continuing to pump liquid. The fitting was then reinverted, and by joggling the pump switch, a drop of liquid was caused to remain hanging from the fitting. The pump was then stopped and the top fitting clamped to a support, taking care not to dislodge the liquid drop hanging from the fitting.

The liquid in the column down to about 1/8 inch above the resin bed face was then removed with a small bulb pipet, care being taken not to disturb the resin bed. The flange seat and top of the column was then carefully cleaned and dried off with facial tissue. The reaction sample, generally 0.250-0.500 ml., was then delivered to the column with the micropipet. This was done slowly and in a manner to avoid disturbing the resin bed surface and not touching the pipet needle to the column walls, especially above the liquid surface. It proved convenient to steady the micropipet in a clamp jig when delivering the sample, starting with the tip

of the pipet needle just into the liquid above the resin and slowly withdrawing the pipet as the sample was delivered. The liquid above the resin was then forced into the resin with nitrogen at 50 p.s.i.g. All of the liquid must go into the resin, but it must not be forced in any farther. This was conveniently done by shutting off the nitrogen supply when the liquid was about 3/16 inch from the resin surface and then letting the level coast in until it was just at the resin surface. While the last portion of the liquid was being driven into the column, the nitrogen fitting holding clamp was removed and the fitting held in place manually so that it could be eased off gently at precisely the time the liquid surface coincided with the resin surface. About 0.55 ml. of 0.12M H_3BO_3 , pH 8.50, was then carefully delivered to the column above the resin. This liquid was also forced into the column with nitrogen as before. The column was then filled to near overflowing with 0.12M H_3BO_3 , pH 8.50, and the top column fitting placed loosely in position. At this point, it was essential to avoid inclusion of any trapped air bubbles in the column system. If necessary, a fresh drop or so of liquid was again forced out of the column top fitting with the micropump just before meshing the hanging drop of liquid with the convex liquid surface at the top of the column. With the help of a vinyl-coated column clamp, the top fitting was then positively forced into the column. The extruded liquid was caught with tissue. A fairly significant back pressure (about 800 p.s.i.g.) should develop as the fitting is forced in. If this back pressure was low, it was almost certain that the micropump check valves were leaking and/or some air bubbles were entrained in the system between the micropump inlet and the top of the resin. Either of these faults was eliminated before proceeding further as they would otherwise cause lack of control in eluent pumping rate, which, for quantitative work, should be as constant as possible. When the column back pressure dropped below 100 p.s.i.g., the vinyl-coated clamp was repositioned and all extruded liquid wiped from around the column top, and a second uncoated clamp placed in position over the coated clamp. The

micropump suction line was then connected to the Autograd. The Autograd stirrer was switched on and the interchamber valves opened in sequence, beginning with the one farthest from the outlet chamber. The micropump was started and the inlet line to the pump watched carefully for a few seconds to determine that the inlet system was free of entrained air bubbles. If any did show up, the pump was stopped and the air cleared from the line. The recorder chart drive was then started and the Auto-Analyzer sample line connected to the bottom of the column. Upon completion of a run, the column was immediately washed for one hour with $0.50M$ $K_2B_4O_7$ and then for one hour with $0.12M$ H_3BO_3 , pH 8.50, by pumping through to the drain with the micropump.

AUTO-ANALYZER PROCEDURES

The Auto-Analyzer was started about one-half hour before it was to be connected to the column. The "instrument" switch and the colorimeter "power" switch were turned on. The proportioning pump sample line was placed into $0.12M$ H_3BO_3 , pH 8.50, solution and the reagent line into the orcinol/ H_2SO_4 solution. The proportioning pump manifold tubing array end block was placed in position on the pump platen alignment pegs and the tubes checked for correct positioning. The roller assembly was swung down into place and checked for firm latching. This positioning of the roller assembly automatically started the proportioning pump. In about 15 minutes, liquid progressed all the way through the Auto-Analyzer system, after which the photoelectric circuitry was adjusted at 0.02 and infinite absorbance. With the "chart drive" switch on and a blank (0) aperture in place between the light source and the colorimeter flow cell, the recorder pen was adjusted to infinite absorbance with the "zero" control. Any air possibly entrained in the flow cell was then cleared by manually pinching off the line from the flow cell to the proportioning pump at a point close to the flow cell and then releasing the pinch suddenly after four air slugs had passed the cell. Sometimes this air clearance

operation had to be repeated. The blank was then removed and the pen adjusted to 0.02 absorbance with the "%T" control. Adjustment to 0.00 absorbance was avoided so that the pen would be free to move to an absorbance lower than 0.02 should the liquid in the flow cell so require. This adjustment procedure was rechecked about fifteen minutes after the sample line was connected to the column to correct for any minor changes in base-line color of the liquid through the flow cell and to ensure that the flow cell was completely cleared of entrained air.

At the completion of a run, the "chart drive," "instrument," and colorimeter "power" switches were turned off. The sample line was disconnected from the column and placed into distilled water. The reagent line was removed from the orcinol/ H_2SO_4 solution, rinsed off by immersion in distilled water, and then placed into a separate container of distilled water. Distilled water was then pumped through the whole system for about fifteen minutes, after which the line ends were lifted out of the distilled water, and the pumping continued until the water in the system was pumped out, which took about 10 minutes. The roller assembly was then unlatched and swung up clear of the tubes. The end block at the suction end of the tubing manifold was lifted from its positioning pegs and laid on the pump platen to permit the tubing to relax under no tension.

APPENDIX III

THE CHROMATOGRAPHY DATA FROM THE REACTION RUNS
AND CONVERSION OF IT TO CONCENTRATIONS OF CARBOHYDRATES

CHROMATOGRAPHY DATA FROM REACTION RUNS

Six series of reaction runs were made for the generation of final kinetic data using the materials and solutions described in Appendix I and the procedures detailed in Appendix II. Reaction Runs 41 (RR 41) were made with glucose as starting material. Then RR 42 with fructose, RR 43 with mannose, RR 44 with cellobiose, RR 47 with cellobiulose, and RR 48 with 4-O- β -D-glucosyl-D-mannose. The reaction conditions for each of these series and the chromatography data obtained are given in Tables IV-IX, respectively.

CALIBRATION OF ABSORBANCE AGAINST WEIGHT OF CARBOHYDRATE

Calibration of chromatography data against weights of carbohydrate materials of interest in the sample applied to the column (and hence concentrations in the corresponding reaction system) was done as follows.

It will be recalled that in colorimetric analysis the absorbance (89, 90) of a colored solution is a dimensionless number and is the logarithm of the ratio of the transmittance of light (at a chosen very narrow wavelength band) through a given path length of an uncolored solution in a given transmittance measuring system to the transmittance through the same system when the solution is colored. In colored solutions for which the Lambert-Beer's law (89, 90) applies the concentration of the color producing material is directly proportional to the absorbance, and hence in such solutions a rectangular plot of absorbance against quantity of the color producing material gives a straight line through the origin. Kesler (70) showed that such plots of the absorbance value at the peak maximum for a number of carbohydrates gave straight lines through the origin when absorbance was measured

TABLE IV

REACTION RUN 41 (GLUCOSE)--CHROMATOGRAPHY DATA

$[G]_0 = 0.00224$; $[OH^-]_0 = 0.9833$; $T, ^\circ C. = 22.20 \pm 0.02$ Absorbance (Abs.) measured at 420 nm.

Reaction Time, hr.	Column Run No.	G_0 Basis, $\mu g.$	Glucose		Mannose		Fructose	
			Abs.	Peak Time, hr.:min.	Abs.	Peak Time, hr.:min.	Abs.	Peak Time, hr.:min.
0.00	133	82.7	0.520	8:05	0	--	0	--
0.00	135	82.7	0.533	8:07	0	--	0	--
0.00	137	68.9	0.454	8:07	0	--	0	--
6.17	140	68.9	0.378	8:20	0.0018	5:40	0.0098	6:55
10.00	155	68.9	0.342	7:52	0.0026	5:25	0.0129	6:32
14.53	134	82.7	0.362	8:05	0.0049	5:25	0.0195	6:43
20.63	148	68.9	0.253	7:55	0.0058	5:25	0.0145	6:36
27.00	138	68.9	0.209	8:09	0.0072	5:29	0.0135	6:44
37.00	136	82.7	0.196	8:10	0.0099	5:29	0.0117	6:47
50.37	139	68.9	0.114	8:10	0.0106	5:29	0.0088	6:45
60.22	174	68.9	0.099	7:55	0.0121	5:20	0.0090	6:35
71.00	152	68.9	0.081	7:56	0.0130	5:27	0.0058	6:37
89.45	141	68.9	0.037	8:14	0.0076	5:35	0.0027	6:47

TABLE V

REACTION RUN 42 (FRUCTOSE)--CHROMATOGRAPHY DATA

$[F]_0 = 0.00218$; $[OH^-]_0 = 0.9833$; $T, ^\circ C. = 22.20 + 0.02$ Absorbance (Abs.) measured at 420 nm.

Reaction Time, hr.	Column Run No.	F ₀ Basis, $\mu g.$	Fructose			Mannose			Glucose		
			Abs.	Peak Time, hr.:min.	Abs.	Peak Time, hr.:min.	Abs.	Peak Time, hr.:min.	Abs.	Peak Time, hr.:min.	
0.00	142	66.6	0.0811	6:52	0	--	0	--	0	--	
4.82	170	66.6	0.0465	6:33	0.0053	5:20	0.0533	7:55			
8.13	145	66.6	0.0370	6:42	0.0072	5:25	0.0740	8:05			
11.00	143	66.6	0.0282	6:55	0.0086	5:37	0.0862	8:21			
14.98	171	66.6	0.0237	6:35	0.0090	5:20	0.0832	8:04			
18.12	147	66.6	0.0153	6:36	0.0103	5:27	0.0912	7:57			
24.60	144	66.6	0.0112	6:58	0.0116	5:39	0.0882	8:23			
30.15	150	66.6	0.0081	6:37	0.0112	5:26	0.0849	7:58			
37.00	172	66.6	0.0067	6:30	0.0113	5:15	0.0695	7:50			
46.42	151	66.6	0.0049	6:38	0.0111	5:25	0.0594	7:56			
71.43	149	66.6	0.0022	6:32	0.0098	5:25	0.0344	7:55			

TABLE VI

REACTION RUN 43 (MANNOSE)---CHROMATOGRAPHY DATA

 $[M]_0 = 0.00214$; $[OH^-]_0 = 0.9833$; $T, ^\circ C. = 22.20 \pm 0.02$ Absorbance (Abs.) measured at 420 nm.

Reaction Time, hr.	Column Run No.	M_0 Basis, $\mu g.$	Mannose		Fructose		Glucose	
			Abs.	Peak Time, hr.:min.	Abs.	Peak Time, hr.:min.	Abs.	Peak Time, hr.:min.
0.00	159	65.5	0.276	5:25	0	--	0	--
0.00	165	65.5	0.267	5:22	0	--	0	--
0.00	173	68.2	0.299	5:15	0	--	0	--
5.95	160	65.5	0.268	5:25	0.0031	6:35	0.0045	7:52
12.03	168	65.5	0.258	5:21	0.0040	6:35	0.0089	7:57
12.03	177	98.9	0.336 ^a	5:14	0.0063	6:30	0.0135	7:51
24.00	164	65.5	0.213	5:31	0.0045	6:47	0.0192	8:09
24.00	178	131.8	0.349 ^a	5:13	0.0121	6:30	0.0392	7:50
37.20	176	135.9	0.292 ^a	5:11	0.0131	6:29	0.0581	7:51
48.00	163	65.5	0.160	5:22	0.0049	6:37	0.0342	7:56
48.00	179	131.8	0.231 ^a	5:13	0.0121	6:30	0.0582	7:50
58.30	167	65.5	0.146	5:22	0.0053	6:38	0.0346	7:59
78.00	169	65.5	0.112	5:21	0.0049	6:35	0.0301	7:55
96.00	162	65.5	0.079	5:24	0.0027	6:35	0.0254	7:52

^a

These values not used. These repeat runs with larger samples made to emphasize F and G peaks.

TABLE VII

REACTION RUN 44 (CELLOBIOSE)--CHROMATOGRAPHY DATA

$[GG]_0 = 0.00119$; $[OH^-]_0 = 0.9845$; $T, ^\circ C. = 22.20 + 0.02$ Absorbance (Abs.) measured at 420 nm.

Reaction Time, hr.	Column Run No.	GG ₀ Basis, $\mu g.$	Cellobiose		Glucosylmannose		Cellobiulose	
			Abs.	Peak Time, hr.:min.	Abs.	Peak Time, hr.:min.	Abs.	Peak Time, hr.:min.
3.45	195	48.4	0.243	1:17	Trace	4:15	0.0305	4:41
6.00	197	48.4	0.202	1:25	Trace	4:20	0.0463	4:55
10.00	199	69.1	0.232	1:20	0.0036	4:15	0.0767	4:50
10.68	198	53.3	0.184	1:20	0.0031	4:25	0.0659	4:59
17.73	196	69.1	0.147	1:17	0.0058	4:10	0.0771	4:42
26.08	201	96.6	0.140	1:18	0.0075	4:15	0.0840	4:51
38.80	204	110.5	0.084	1:17	0.0081	4:05	0.0558	4:43
48.13	203	96.6	0.037	1:20	0.0080	4:08	0.0350	4:43
71.00	200	96.6	0.019	1:17	0.0071	4:10	0.0153	4:47

Reaction Time, hr.	Column Run No.	Mannose		Fructose		Glucose	
		Abs.	Peak Time, hr.:min.	Abs.	Peak Time, hr.:min.	Abs.	Peak Time, hr.:min.
3.45	195	0		0		0.0085	7:46
6.00	197	0		0		0.0152	8:05
10.00	199	0		0		0.0355	8:00
10.68	198	0		0		0.0311	8:10
17.73	196	0		0		0.0595	7:49
26.08	201	Trace	5:23	0.0044	6:40	0.1140	8:06
38.80	204	Trace	5:20	0.0076	6:33	0.1250	7:55
48.13	203	0.0027	5:14	0.0072	6:29	0.1020	7:50
71.00	200	0.0053	5:18	0.0053	6:35	0.0811	7:59

TABLE VIII

REACTION RUN 47 (CELLOBIULOSE)--CHROMATOGRAPHY DATA

$[GF]_O = 0.00080$; $[OH^-]_O = 0.9845$; $T, ^\circ C. = 22.20 \pm 0.02$ Absorbance (Abs.) measured at 420 nm.

Reaction Time, hr.	Column Run No.	GF Basis, $\mu g.$	Cellobiulose		Cellobiulose		Glucosylmannose		
			Abs.	Peak Time, hr.:min.	Abs.	Peak Time, hr.:min.	Abs.	Peak Time, hr.:min.	
2.00	210	46.7	0.137	4:46	0.0107	1:20	0	--	
4.00	211	65.5	0.155	4:43	0.0250	1:17	0	--	
8.55	212	93.4	0.129	4:42	0.0483	1:20	Trace	4:05	
12.00	213	93.4	0.0980	4:43	0.0507	1:20	0.0036	4:06	
16.13	223	102.9	0.0843	4:40	0.0528	1:16	0.0076	4:00	
22.00	225	93.4	0.0538	4:39	0.0456	1:15	0.0044	3:54	
26.62	215	93.4	0.0404	4:40	0.0404	1:17	0.0049	3:55	
36.28	214	93.4	0.0193	4:44	0.0287	1:18	Trace	4:12	
Mannose									
2.00	210		0						Glucose
4.00	211		0				0.0200	7:55	
8.55	212		0				0.0430	7:53	
12.00	213		0			6:30	0.0993	7:52	
16.13	223		0			6:30	0.1150	7:52	
22.00	225		--				--		
26.62	215		0.0022	5:10	0.0062	6:28	0.1328	7:50	
36.28	214		0.0018	5:13	0.0063	6:30	0.1190	7:53	
			0.0026	5:14	0.0058	6:30	0.0990	7:52	

TABLE IX

REACTION RUN 48 (GLUCOSYLMANNOSE)--CHROMATOGRAPHY DATA

$[GM]_0 = 0.00110$; $[OH^-]_0 = 0.9845$; $T, ^\circ C. = 22.20 \pm 0.02$ Absorbance (Abs.) measured at 420 nm.

Reaction Time, hr.	Column Run No.	Glucosylmannose			Cellobiose			Cellobiulose																																																																														
		GM ₀ Basis, $\mu g.$	Abs.	Peak Time, hr.:min.	Abs.	Peak Time, hr.:min.	Abs.	Peak Time, hr.:min.																																																																														
6.00	248	42.2	0.152	4:10	0.0045	1:20	0.0081	4:46																																																																														
12.10	245	48.2	0.152	4:10	0.0088	1:18	0.0142	4:47																																																																														
12.10	252	42.2	0.139	4:08	0.0085	1:22	0.0108	4:45																																																																														
24.83	246	60.3	0.132	4:09	0.0157	1:19	0.0194	4:45																																																																														
24.83	253	60.3	0.147	4:07	0.0149	1:20	0.0197	4:44																																																																														
36.00	247	72.3	0.124	4:11	0.0184	1:19	0.0221	4:47																																																																														
36.00	257	42.2	0.084	4:10	0.0076	1:18	0.0149	4:44																																																																														
48.18	250	84.4	0.096	4:03	0.0266	1:16	0.0244	4:40																																																																														
48.18	254	120.6	0.112	3:55	0.0336	1:17	0.0308	4:40																																																																														
49.00	256	42.2	0.081	4:08	0.0130	1:17	0.0157	4:43																																																																														
<table border="1"> <thead> <tr> <th colspan="3"></th> <th>Mannose</th> <th>Fructose</th> <th>Glucose</th> </tr> </thead> <tbody> <tr> <td>6.00</td> <td>248</td> <td>0</td> <td>0</td> <td>0</td> <td>0.0031</td> <td>7:55</td> </tr> <tr> <td>12.10</td> <td>245</td> <td>0</td> <td>0</td> <td>0</td> <td>0.0088</td> <td>7:53</td> </tr> <tr> <td>12.10</td> <td>252</td> <td>--</td> <td>--</td> <td>--</td> <td>--</td> <td>--</td> </tr> <tr> <td>24.83</td> <td>246</td> <td>0</td> <td>0</td> <td>0</td> <td>0.0204</td> <td>7:53</td> </tr> <tr> <td>24.83</td> <td>253</td> <td>--</td> <td>--</td> <td>--</td> <td>--</td> <td>--</td> </tr> <tr> <td>36.00</td> <td>247</td> <td>0</td> <td>0</td> <td>0</td> <td>0.0291</td> <td>7:56</td> </tr> <tr> <td>36.00</td> <td>257</td> <td>Trace</td> <td>Trace</td> <td>Trace</td> <td>0.0209</td> <td>7:52</td> </tr> <tr> <td>48.18</td> <td>250</td> <td>Trace</td> <td>Trace</td> <td>Trace</td> <td>0.0495</td> <td>7:50</td> </tr> <tr> <td>48.18</td> <td>254</td> <td>0.0018</td> <td>0.0027</td> <td>0.0027</td> <td>0.0695</td> <td>7:49</td> </tr> <tr> <td>49.00</td> <td>256</td> <td>Trace</td> <td>Trace</td> <td>Trace</td> <td>0.0265</td> <td>7:47</td> </tr> </tbody> </table>														Mannose	Fructose	Glucose	6.00	248	0	0	0	0.0031	7:55	12.10	245	0	0	0	0.0088	7:53	12.10	252	--	--	--	--	--	24.83	246	0	0	0	0.0204	7:53	24.83	253	--	--	--	--	--	36.00	247	0	0	0	0.0291	7:56	36.00	257	Trace	Trace	Trace	0.0209	7:52	48.18	250	Trace	Trace	Trace	0.0495	7:50	48.18	254	0.0018	0.0027	0.0027	0.0695	7:49	49.00	256	Trace	Trace	Trace	0.0265	7:47
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by the procedures and equipment used in this research. This finding was thoroughly examined in this study and by additional other studies in these laboratories and found to be valid providing quantities were adjusted to give absorbance values below about 0.400. This straight line relationship was accepted and used in this study for calibrating chromatography data and for converting it to concentrations of carbohydrates in reaction systems. Typical data obtained while exploring this straight line relationship are given in Table X and plotted in Fig. 16.

TABLE X

TYPICAL ABSORBANCE VS. WEIGHT DATA
FROM CHROMATOGRAPHY CALIBRATION STUDIES

Column Run Number	Wt. Applied, $\mu\text{g.}$	Abs., 420 nm.	Peak Time, hr.:min.
Glucose			
28	305	0.351	6:47
29	122	0.149	6:46
30	122	0.150	6:47
31	488	0.541	6:45
32	403	0.451	6:46
34	244	0.282	6:47
Cellobiose			
53	174	0.385	1:15
55	254	0.570	1:14
58	102	0.241	1:15
59	214	0.503	1:15

Chromatograms for calibration of kinetic data were run from solutions (boiled, N_2 -saturated distilled water, 0.33M in NaCl and 0.12M in H_3BO_3 at pH 7-8) containing several of the carbohydrates each at 0.0050-0.0100 \pm 0.00005 g./100.0 ml. and from the 0.00 hr. solutions from reaction runs. Data from these calibration

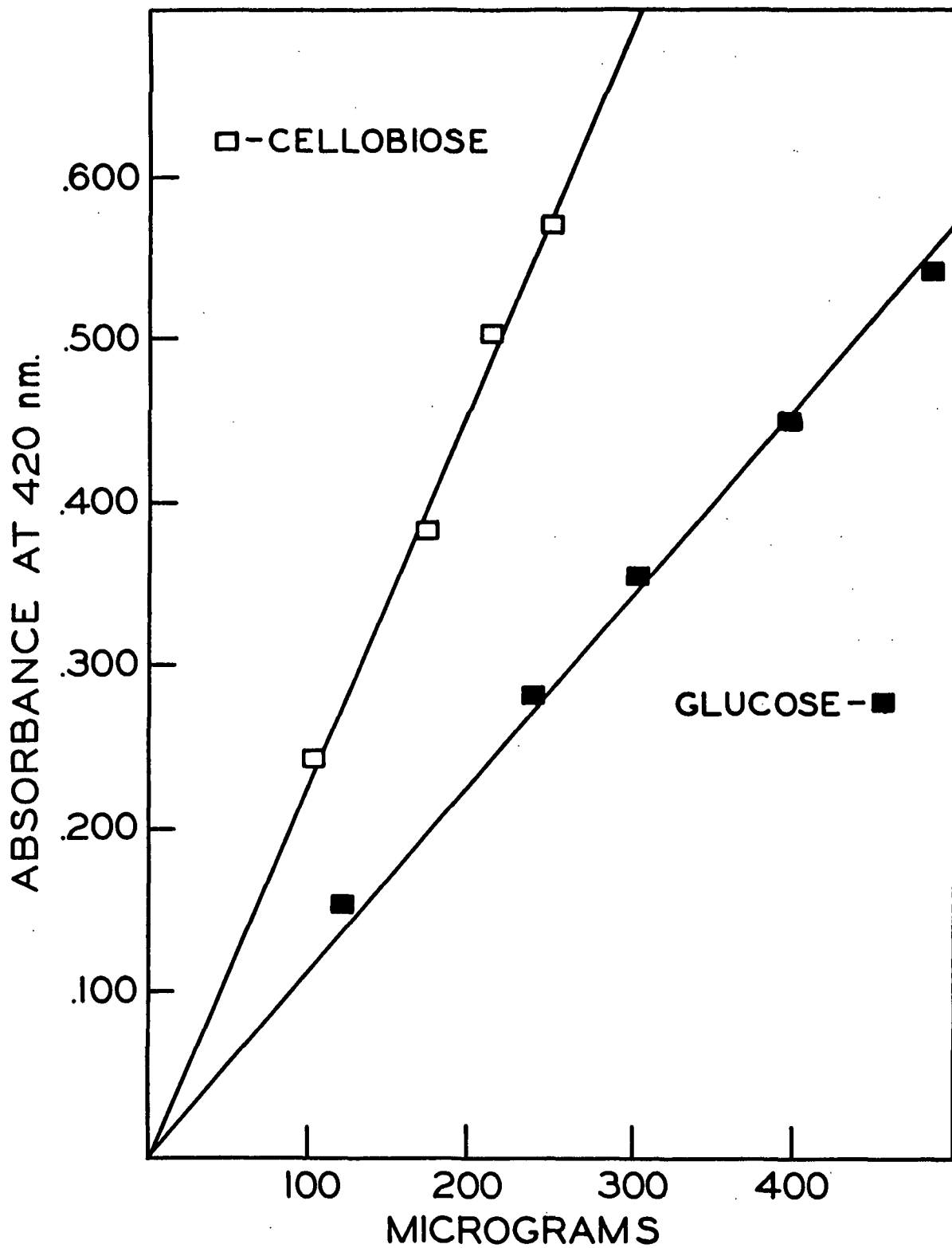


Figure 16. Typical Calibration Plots.

chromatograms are given in Tables XI and XII. Large-scale plots of these data (absorbance vs. $\mu\text{g.}$ carbohydrate on a 500-mm. x 500-mm. plot) were made and best-fit (visual) straight lines drawn through the origin and the experimental points. From these plots the absorbance vs. weight relationships given in Tables XIII and XIV were read off and these then used to convert the chromatography data of the reaction runs (Tables IV through IX) to the relative molar data given in Tables XV-XX, respectively, for the reaction runs with glucose, fructose, mannose, cellobiose, cellobiulose, and 4-O- β -D-glucosyl-D-mannose as starting material. The conversion factors in Table XIV include molecular weight factors based on a molecular weight of 342.3 for the disaccharides and of 180.16 for the monomers.

The relative molar data of Tables XV through XX were used to generate reaction rate constants as described in Appendix IV and to obtain the plots of relative molarities vs. reaction time which are shown as Fig. 7 through 13 in the main body of this report.

TABLE XI

CHROMATOGRAPHY CALIBRATION DATA FOR REACTION RUNS 41, 42, AND 43

Column Run No.	Glucose			Fructose			Mannose		
	Applied, $\mu\text{g.}$	Abs. hr.:min.	Peak Time, hr.:min.	Applied, $\mu\text{g.}$	Abs. hr.:min.	Peak Time, hr.:min.	Applied, $\mu\text{g.}$	Abs. hr.:min.	Peak Time, hr.:min.
131	54.3	0.405	8:10	52.9	0.077	6:47	23.1	0.125	5:31
132	26.5	0.205	8:10	25.9	0.035	6:46	11.1	0.064	5:30
133	82.7	0.520	8:05	0			0		
135	82.7	0.533	8:07	0			0		
137	68.9	0.454	8:07	0			0		
142	0			66.6	0.081	6:52	0		
153	66.3	0.451	8:03	64.1	0.081	6:36	63.4	0.295	5:26
154	66.3	0.451	7:53	64.1	0.077	6:35	63.4	0.294	5:27
156	33.2	0.245	7:52	32.1	0.035	6:32	31.7	0.160	5:27
157	33.2	0.230	7:51	32.1	0.038	6:31	31.7	0.155	5:25
158	66.3	0.454	7:52	64.1	0.092	6:31	63.4	0.289	5:25
159	0			0			65.5	0.276	5:25
165	0			0			65.5	0.267	5:22
173	0			0			68.2	0.299	5:15

TABLE XII

CHROMATOGRAPHY CALIBRATION DATA FOR REACTION RUNS 44, 47, AND 48

Column Run No.	Cellobiiose			Cellobiulose			Glucosylmannose		
	Applied, µg.	Abs.	Peak Time, hr.:min.	Applied, µg.	Abs.	Peak Time, hr.:min.	Applied, µg.	Abs.	Peak Time, hr.:min.
190	69.1	0.448	1:19	0			0		
191	48.4	0.325	1:19	0			0		
192	48.4	0.333	1:16	0			0		
193	48.4	0.324	1:18	0			0		
194	20.7	0.141	1:17	0			0		
207	0			46.7	0.169	4:46	0		
208	0			46.7	0.178	4:49	0		
209	0			28.0	0.113	4:47	0		
216	0			46.7	0.180	4:45	0		
244	0			0			48.3	0.184	4:12
249	0			0			54.3	0.212	4:08
251	0			0			42.2	0.162	4:10
255	0			0			42.2	0.170	4:08

TABLE XIII

ABSORBANCE-WEIGHT CONVERSION FACTORS BETWEEN
GLUCOSE (G), FRUCTOSE (F), AND MANNOSE (M)
IN CHROMATOGRAPHY DATA FROM REACTION RUNS 41, 42, AND 43

For a given reaction system and on the basis of the same chromatogram sample size for \underline{X} and \underline{Y}_0 , multiply the absorbance of \underline{X} by the factors below and by 100 to obtain the quantity of \underline{X} in a reaction system expressed as the percent relative molarity of \underline{Y}_0 in that reaction system.

\underline{X}	Glucose	Fructose	Mannose
Calibrated absorbance at 50 μ g.	0.340	0.061	0.220
\underline{Y}_0			
G_0	1/abs. G_0	5.574/abs. G_0	1.545/abs. G_0
F_0	0.1794/abs. F_0	1/abs. F_0	0.2773/abs. F_0
M_0	0.6470/abs. M_0	3.606/abs. M_0	1/abs. M_0

TABLE XIV

ABSORBANCE-WEIGHT CONVERSION FACTORS BETWEEN GLUCOSE, FRUCTOSE, MANNOSE
CELLOBIOSE (GG), CELLOBIULOSE (GF), AND GLUCOSYLMANNOSE (GM),
IN CHROMATOGRAPHY DATA FROM REACTION RUNS 44, 47, AND 48

For a given reaction system and on the basis of the same chromatogram sample size for \bar{X} and \bar{Y}_O , multiply the absorbance of \bar{X} by the factors below and by 100 to obtain the quantity of \bar{X} in a reaction system expressed as the percent relative molarity of \bar{Y}_O in that reaction system.

\bar{X}	Glucose	Fructose	Mannose	Cellobiose	Cellobiulose	Glucosylmannose
Calibrated absorbance at 50 μ g.	0.340	0.061	0.220	0.338	0.192	0.195
\bar{Y}_O						
GG _O	1.889/abs. GG _O	10.53 /abs. GG _O	2.920/abs. GG _O	1/abs. GG _O	1.763 abs./GG _O	1.737 /abs. GG _O
GF _O	1.071/abs. GF _O	5.972/abs. GF _O	1.656/abs. GF _O	0.5671/abs. GF _O	1/abs. GF _O	0.9849/abs. GF _O
GM _O	1.073/abs. GM _O	6.064/abs. GM _O	1.681/abs. GM _O	0.5758/abs. GM _O	1.015/abs. GM _O	1/abs. GM _O

TABLE XV

REACTION RUN 41 (GLUCOSE)--
RELATIVE CARBOHYDRATE PERCENT MOLARITIES

$[G]_0 = 0.00224$; $[OH^-]_0 = 0.9833$; $T, ^\circ C. = 22.20 \pm 0.02$

Reaction Time, hr.	Glucose, %M G_0	Mannose, %M G_0	Fructose, %M G_0
0.00	100.0	0.0	0.0
6.17	81.2	0.6	11.7
10.00	73.5	0.9	15.5
14.53	64.7	1.4	19.5
20.63	54.3	2.0	17.4
27.00	44.8	2.4	16.2
37.00	34.9	2.7	11.7
50.37	24.4	3.5	10.5
60.22	21.2	4.0	10.8
71.00	17.4	4.3	7.0
89.45	7.9	2.5	3.7

TABLE XVI

REACTION RUN 42 (FRUCTOSE)--
RELATIVE CARBOHYDRATE PERCENT MOLARITIES

$[F]_0 = 0.00218$; $[OH^-]_0 = 0.9833$; $T, ^\circ C. = 22.20 \pm 0.02$

Reaction Time, hr.	Fructose $\%M F_0$	Mannose, $\%M F_0$	Glucose, $\%M F_0$
0.00	100.0	0.0	0.0
4.82	57.4	1.8	11.8
8.13	45.6	2.5	16.4
11.00	34.8	2.9	18.4
14.98	29.3	3.1	18.4
18.12	18.9	3.5	20.2
24.60	13.8	4.0	19.5
30.15	10.0	3.8	18.7
37.00	8.3	3.9	15.4
46.42	6.1	3.8	13.1
71.43	2.7	3.4	7.7

TABLE XVII

REACTION RUN 43 (MANNOSE)--
RELATIVE CARBOHYDRATE PERCENT MOLARITIES

$[M]_0 = 0.00214$; $[OH^-]_0 = 0.9833$; $T_2, ^\circ C. = 22.20 \pm 0.02$

Reaction Time, hr.	Mannose, % \underline{M} M_0	Fructose, % \underline{M} M_0	Glucose, % \underline{M} M_0
0.00	100.0	0.0	0.0
5.95	93.0	3.9	1.0
12.03	89.5	5.0	2.0
12.03	--	5.2	2.0
24.00	73.9	5.6	4.3
24.00	--	7.5	4.4
37.20	--	7.9	6.3
48.00	55.5	6.1	7.6
48.00	--	7.5	6.5
58.30	50.6	6.6	7.7
78.00	38.8	6.1	6.7
96.00	27.4	3.4	5.7

TABLE XVIII

REACTION RUN 44 (CELLOBIOSE) --RELATIVE CARBOHYDRATE PERCENT MOLARITIES

$$[\text{GG}]_0 = 0.00119; [\text{OH}^-]_0 = 0.9845; T, ^\circ\text{C.} = 22.20 \pm 0.02$$

Reaction Time, hr.	Cellobiose, %M GG ₀	Glucosylmannose, %M GG ₀	Cellobinulose, %M GG ₀	Mannose, %M GG ₀	Fructose, %M GG ₀	Glucose, %M GG ₀
0.00	100.0	0.0	0.0	0.0	0.0	0.0
3.45	71.5	Trace	16.5	0.0	0.0	4.9
6.00	62.0	Trace	25.0	0.0	0.0	8.8
10.00	49.6	1.3	29.0	0.0	0.0	14.4
10.68	49.2	1.5	31.2	0.0	0.0	15.8
17.73	31.5	2.1	29.2	0.0	0.0	24.0
26.08	21.4	2.0	22.6	Trace	7.0	33.0
38.80	11.3	1.9	13.2	Trace	10.8	31.6
48.13	5.7	2.1	9.5	1.2	11.5	29.5
71.00	2.9	1.9	4.1	2.7	8.6	23.4

TABLE XIX

REACTION RUN 47 (CELLOBIULOSE)--RELATIVE CARBOHYDRATE PERCENT MOLARITIES

$$[\text{GF}]_0 = 0.00080; [\text{OH}^-]_0 = 0.9845; \bar{T}, \text{ }^\circ\text{C.} = 22.20 \pm 0.02$$

Reaction Time, hr.	Cellobiulose, %M GF ₀	Cellobiose, %M GF ₀	Glucosylmannose, %M GF ₀	Mannose, %M GF ₀	Fructose, %M GF ₀	Glucose, %M GF ₀
0.00	100.0	0.0	0.0	0.0	0.0	0.0
2.00	76.6	3.4	0.0	0.0	0.0	12.0
4.00	62.0	5.7	0.0	0.0	0.0	18.4
8.55	36.0	7.7	Trace	0.0	3.0	29.8
12.00	27.4	8.0	1.0	0.0	5.3	34.4
16.13	21.4	7.6	1.9	--	--	--
22.00	15.0	7.2	1.2	1.0	10.5	39.7
26.62	11.3	6.4	1.4	0.8	11.0	35.6
36.28	5.4	4.6	--	1.2	9.7	29.6

TABLE XX

REACTION RUN 48 (GLUCOSYLMANNOSE) --RELATIVE CARBOHYDRATE PERCENT MOLARITIES

$[GM]_O = 0.00110$; $[OH^-]_O = 0.9845$; $T, ^\circ C. = 22.20 \pm 0.02$

Reaction Time, hr.	Glucosylmannose, %M GM _O	Cellobiose, %M GM _O	Cellobiulose, %M GM _O	Mannose, %M GM _O	Fructose, %M GM _O	Glucose, %M GM _O
0.00	100.00	0.0	0.0	0.0	0.0	0.0
6.00	92.5	1.6	5.0	0.0	0.0	2.0
12.10 ^a	82.8	2.8	7.2	0.0	0.0	5.0
24.83 ^a	59.4	3.8	8.5	0.0	0.9	9.3
36.00 ^a	47.5	3.3	8.6	Trace	Trace	12.4
48.18 ^a	26.5	4.4	7.1	0.6	2.0	16.1

^a These values at these times are the average values of the data converted from Table IX.

APPENDIX IV

DERIVATION OF THE REACTION RATE CONSTANTS
FROM THE EXPERIMENTAL DATA

The derivation of the rate constants prevailing in the chosen reaction system of cellobiose in aqueous sodium hydroxide proceeded through a stepwise plan. First, the rate constants in the monomer portion of the system (glucose-fructose-mannose) were approximated by graphical and algebraic means from the plots of the relative molarities vs. time (Fig. 12) and Equations (1)-(4) on the following page. These approximate values were then refined to yield best fit curves by reiterative solution of the rate Equations (1)-(4) using a digital analog-simulator program (PACTOLUS), described in Appendix V, on an IBM 1620 computer equipped with a Calcomp 565 Digital Incremental Plotter. This same sequence was then applied to the disaccharide portion of the system (cellobiose, cellobiulose, and glucosylmannose). The final step was an extended refinement of all the rate constants together by reiterative solution through the computer using a program configuration based on the general case of the overall reaction scheme for the transformation and degradation of cellobiose in aqueous sodium hydroxide as given in Fig. 6.

This general case reaction scheme was based on the reversible aldo-keto isomerizations, C2 epimerizations, and the 8-1 reaction, experimentally observed in the reaction system. The 1-5, 2-4, 3-6, 7-10, 9-11, and 8-12 reactions were initially included as possibilities and their existence subsequently all established by solution of the applicable rate equations to yield positive rate constants in each case. A, B, C, D, E, and H are any materials other than glucose, fructose, mannose, cellobiose, cellobiulose, and glucosylmannose.

In each of the six reaction run series, plots of the logarithm of the relative concentration of the starting material against time gave quite good straight lines during the early stages of the reaction. From this observation and the experimental

design which provided essentially no change in concentration of the base during the disappearance of at least half the starting material; it was concluded that the several reactions involving the starting material were pseudo-first-order.

For the above reaction system, with first-order reactions, the following equations apply:

$$dG/dt = k_{81}GF + k_{21}F + k_{31}M - (k_{12} + k_{13} + k_{15})G \quad (1)$$

$$dF/dt = k_{12}G + k_{32}M - (k_{21} + k_{23} + k_{24})F \quad (2)$$

$$dM/dt = k_{13}G + k_{23}F - (k_{31} + k_{32} + k_{36})M \quad (3)$$

$$dA/dt = k_{24}F \quad (4)$$

$$dB/dt = k_{15}G \quad (5)$$

$$dC/dt = k_{36}M \quad (6)$$

$$dGG/dt = k_{87}GF + k_{97}GM - (k_{78} + k_{79} + k_{7,10})GG \quad (7)$$

$$dGF/dt = k_{78}GG + k_{98}GM - (k_{81} + k_{87} + k_{89} + k_{8,12})GF \quad (8)$$

$$dGM/dt = k_{79}GG + k_{89}GF - (k_{97} + k_{98} + k_{9,11})GM \quad (9)$$

$$dD/dt = k_{7,10}GG \quad (10)$$

$$dE/dt = k_{9,11}GM \quad (11)$$

$$dH/dt = k_{8,12}GF \quad (12)$$

For the monomer system, large-scale plots (500 mm. x 500 mm.) were made of the relative molarity data given in Tables XV, XVI, and XVII. The slopes of the several curves on the large-scale plots were then obtained by graphical means at selected reaction times.

The graphical technique used for obtaining these slopes (first derivatives) of the concentration vs. time curves was as follows. Two 250-mm. lengths of clear and uniform 10-mm. diameter glass rod were taped snugly together, in parallel arrangement with ends even, using a few wraps of clear Scotch tape. A sharp black-ink ordinate line was drawn through the curves at the selected times. The pair of glass rods was laid across a curve at a point where the slope was to be measured. Then the curve line and the black ordinate line were viewed through the pair of glass rods from directly above the point of interest. Then the position of the glass rods was adjusted slowly until simultaneously (1) the curve appeared a continuous line, and (2) the two wavy, discontinuous segments of the black ordinate line were equidistant from the curve. When the rods were thus positioned, their long axis was parallel with the normal to the tangent to the curve at the point of intersection of the curve and the black ordinate. A conveniently placed line was drawn on the squared paper of the plot to be parallel to this tangent, using a pair of large-sized set squares to transfer the tangent direction. The data thus obtained are summarized in Table XXI.

The data in Table XXI were used in algebraic manipulation of Equations (I)-(IV) in seeking values of the various rate constants. This step was not too successful, and ambiguous results were obtained depending upon the sets of equations solved. In retrospect, it was concluded that the solution of these simultaneous equations is very sensitive to any even rather small inconsistencies in rates and/or concentrations as derived from the data. However, by using the apparent best values of the rate constants obtained through algebraic manipulation as a starting point, it was possible to obtain a set of rate constants through the computer that gave computer drawn plots of relative molarities vs. time which were in very good agreement with the appropriate experimental data as shown in Fig. 12 in the main body of this report.

TABLE XXI

RELATIVE CONCENTRATION AND RATE OF CHANGE OF RELATIVE
CONCENTRATION OF CARBOHYDRATES

Starting Material	Reaction Time, hr.	Relative Conc., moles/mole of starting material			Change of Relative Concentration, moles/mole of starting material/hr.		
		G	F	M	$\frac{dG}{dt}$	$\frac{dF}{dt}$	$\frac{dM}{dt}$
G	0	1.000	0	0	-0.035	--	--
G	5	0.842	0.101	0.006	-0.02439	+0.01575	+0.0009523
G	9	0.749	0.150	0.010	-0.02041	+0.009756	+0.0009523
G	13	0.673	0.179	0.014	-0.01770	+0.005277	+0.0009523
F	0	0	1.000	0	--	-0.10	--
F	4	0.104	0.666	0.015	+0.01923	-0.06897	+0.002647
F	7	0.150	0.486	0.022	+0.01242	-0.04762	+0.002356
F	12	0.195	0.312	0.031	+0.00493	-0.02532	+0.001339
M	0	0	0	1.000	--	--	-0.012
M	8	0.013	0.041	0.909	+0.001862	+0.003745	-0.01124
M	14	0.025	0.060	0.844	+0.001773	+0.002882	-0.01087
M	21	0.039	0.075	0.771	+0.001745	+0.001475	-0.00976
M	21	0.051	0.078	0.703	+0.001629	0.0000	-0.00901

This same sequence was repeated for the disaccharide portion of the overall system and then the rate constants for the whole system refined on the computer. Figures 7 and 8 in the main body of this report are computer drawn plots of relative molarity vs. time relationships for the several disaccharide reaction runs using the final rate constant values obtained which are given in Table I. Some implications of these rate constants are discussed in the main body of this report.

APPENDIX V

THE COMPUTER PROGRAM

Initial values for the rate constants in the rate equations were obtained by a combination of graphical and numerical means as discussed in Appendix IV.

These initial values were then tested by comparing computer integrated rate equation curves with the experimental concentration vs. time data. The values of the rate constants used in the computer program were then slowly adjusted until a set of best fit integrated curves was obtained. This was a fairly long and tedious process, but it was feasible and provided a solution to the problem of abstracting the desired rate constants from the experimental data. It appears* that a direct numerical solution to this problem, via say matrix algebra or a least squares analysis, could be done but with no better result than was obtained by the methods used. It is noted though that any valid method of solution to this set of equations will yield a unique solution (91).

The equipment used in this reiterative process of solution was an IBM 1620 computer coupled to a Calcomp 565 Digital Incremental Plotter. The computer program was PACTOLUS, a digital analog-simulator program written by R. D. Brennan and H. Sano of the International Business Machines Corporation. The wiring diagram blocks established for use with the program are shown in Fig. 17 and 18. The configuration specification used is listed in Table XXII and the initial conditions and parameters in Table XXIII.

*A conclusion based on consultation with and advice from Dr. R. W. Nelson of the faculty of The Institute of Paper Chemistry.

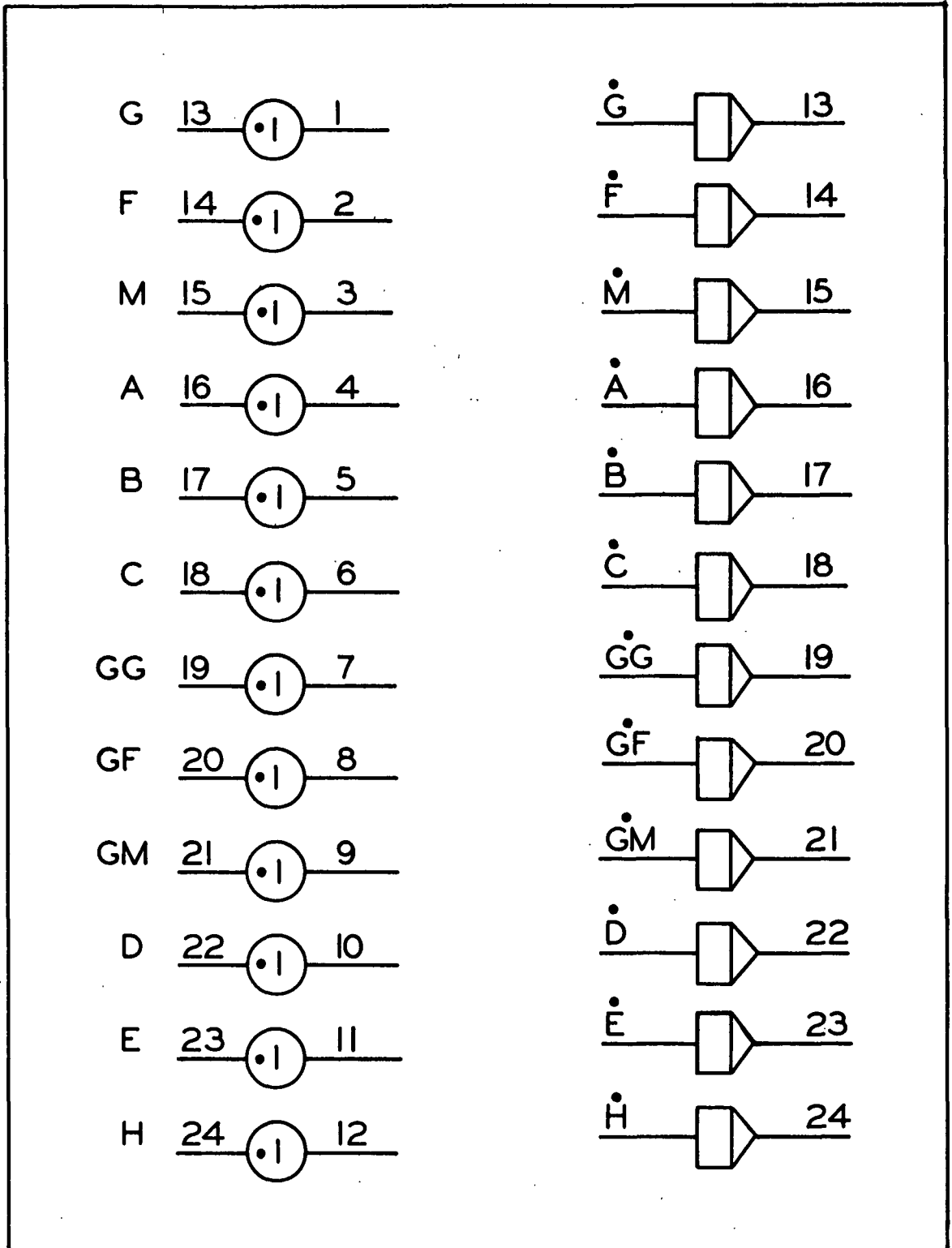


Figure 17. Computer Program Block Diagrams

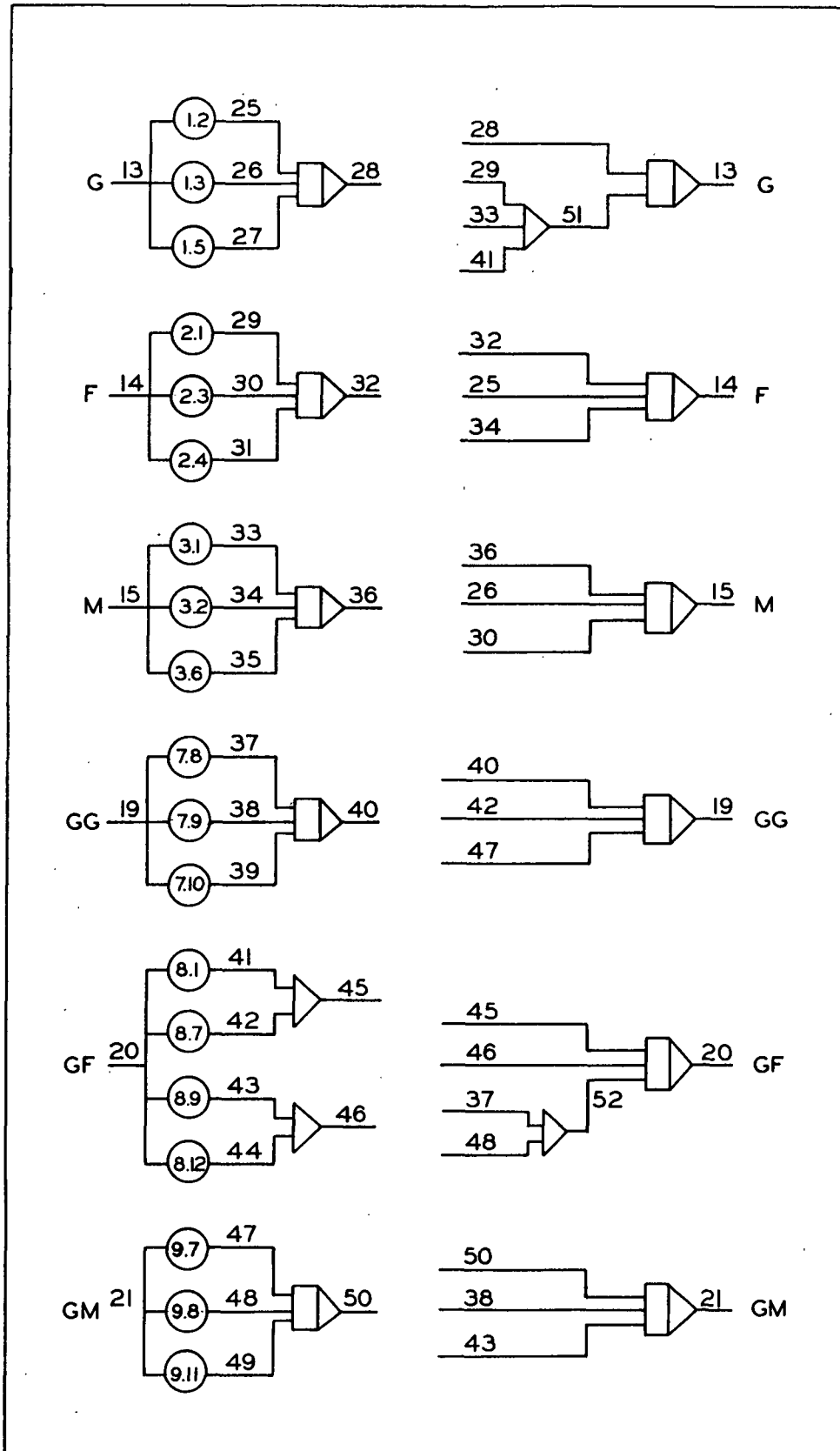


Figure 18. Computer Program Block Diagrams

TABLE XXII

PACTOLUS DIGITAL ANALOG SIMULATOR PROGRAM

Block	Configuration Specification			
	Type	Input 1	Input 2	Input 3
1	G	13	0	0
2	G	14	0	0
3	G	15	0	0
4	G	16	0	0
5	G	17	0	0
6	G	18	0	0
7	G	19	0	0
8	G	20	0	0
9	G	21	0	0
10	G	22	0	0
11	G	23	0	0
12	G	24	0	0
13	I	28	51	0
14	I	32	25	34
15	I	36	26	30
16	I	31	0	0
17	I	27	0	0
18	I	35	0	0
19	I	40	42	47
20	I	45	46	52
21	I	50	38	43
22	I	39	0	0
23	I	49	0	0
24	I	44	0	0
25	G	13	0	0
26	G	13	0	0
27	G	13	0	0
28	+	-25	-26	-27
29	G	14	0	0
30	G	14	0	0
31	G	14	0	0
32	+	-29	-30	-31
33	G	15	0	0
34	G	15	0	0
35	G	15	0	0
36	+	-33	-34	-35
37	G	19	0	0
38	G	19	0	0
39	G	19	0	0
40	+	-37	-38	-39
41	G	20	0	0
42	G	20	0	0
43	G	20	0	0
44	G	20	0	0

TABLE XXII (Continued)

PACTOLUS DIGITAL ANALOG SIMULATOR PROGRAM

Block	Configuration Specification			
	Type	Input 1	Input 2	Input 3
45	+	-41	-42	0
46	+	-43	-44	0
47	G	21	0	0
48	G	21	0	0
49	G	21	0	0
50	+	-47	-48	-49
51	+	29	33	41
52	+	37	48	0
53	G	61	0	0

TABLE XXIII

PACTOLUS DIGITAL ANALOG SIMULATOR PROGRAM

Block	Initial Conditions and Parameters		
	IC/Par. 1	Par. 2	Par. 3
1	0.10000	0.00000	0.00000
2	0.10000	0.00000	0.00000
3	0.10000	0.00000	0.00000
4	0.10000	0.00000	0.00000
5	0.10000	0.00000	0.00000
6	0.10000	0.00000	0.00000
7	0.10000	0.00000	0.00000
8	0.10000	0.00000	0.00000
9	0.10000	0.00000	0.00000
10	0.10000	0.00000	0.00000
11	0.10000	0.00000	0.00000
12	0.10000	0.00000	0.00000
13	100.00000	1.00000	1.00000
14	0.00000	1.00000	1.00000
15	0.00000	1.00000	1.00000
16	0.00000	1.00000	1.00000
17	0.00000	1.00000	1.00000
18	0.00000	1.00000	1.00000
19	0.00000	1.00000	1.00000
20	0.00000	1.00000	1.00000
21	0.00000	1.00000	1.00000
22	0.00000	1.00000	1.00000
23	0.00000	1.00000	1.00000
24	0.00000	1.00000	1.00000
25	0.03600	0.00000	0.00000
26	0.00050	0.00000	0.00000
27	0.00200	0.00000	0.00000
29	0.03800	0.00000	0.00000
30	0.00600	0.00000	0.00000
31	0.07200	0.00000	0.00000
33	0.00050	0.00000	0.00000
34	0.01100	0.00000	0.00000
35	0.00200	0.00000	0.00000
37	0.07800	0.00000	0.00000
38	0.00050	0.00000	0.00000
39	0.00200	0.00000	0.00000
41	0.06500	0.00000	0.00000
42	0.02200	0.00000	0.00000
43	0.00300	0.00000	0.00000
44	0.02300	0.00000	0.00000
47	0.00200	0.00000	0.00000
48	0.01300	0.00000	0.00000
49	0.00600	0.00000	0.00000
53	0.10000	0.00000	0.00000

