FRACTIONATION OF STARCH DEXTRINS USING SEPHADEX

by

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INTRODUCTION

Since the introduction of Sephadex by Porath and Flodin, gel filtration has in a short time become an important separation method. A series of cross linked dextrans marketed under the name of Sephadex, G-10, G-15, G-25, G-50, G-75, G-100, and G-200 are available. These differ in the degree of cross linking and fractionation range. The principle of fractionation is sieving and thus the most highly crossed linked sephadexes such as G-10 fractionate over a low molecular weight range and are applicable for example to the separation of glucose from maltose. The higher numbers such as G-100 and G-200 are applicable to molecular weight polymers of 200,000 or more.

The widest application of Sephadex has probably been in proteins. Applications to carbohydrates have been restricted mainly to sephadexes from G-10 to G-25. These are reported to separate some oligosaccharides. Very few applications of G-75, G-100, and G-200 to polysaccharies can be found.

Starch dextrins prepared by enzymatic or acid hydrolysis are widely used in industry. The spectrum of molecular weight species may vary considerably and in fact a certain molecular weight range may be desirable for a particular function. There is no good method for measuring this parameter quantitatively. It was the purpose of this investigation to determine whether sephadex G-100 and G-200 could be effectively used for this purpose.

LITERATURE CITED

Separations with Sephadex

Principles

The rate of diffusion of dissolved substance in an uncharged, inert three-dimensional medium is related to both the molecular size of the diffusing species and the pore size of the matrix. This phenomenon forms the principle of the molecular sieve effect (1), which is however affected by the presence of ionic groups on the sieve material, such as in ion exchange resins, or the interaction of the diffusing substance with the network by either complex formation or adsorption. Practically, it is of importance that the sieve be in the form of definite particles, or in the form of a membrane. The most suitable substance for the former application is a cross-linked dextran, marketed in different pore sizes under the name "Sephadex". The method is similar to that of column chromatography and has been called "gel filtration" (2).

Since the introduction of sephadex by Porath and Flodin (3), gel filtration has in a short time become an important separation method. It has been widely applied to desalting colloids and separating mixtures of proteins, peptides, and amino acids. Separation of oligosaccharides (4) and fractionation of low-molecular weight dextrans (5) have been performed on the early types of sephadex (G-25 to G-75).

The gel material, sephadex, consists of granules or beads of cross-linked dextran. By reaction with epichlorohydrin, the formation of 1',3'-glyceryl-bridges was possible, and the dextran chains are linked in a three-dimensional network with high content of hydroxyl groups. Although sephadex is water insoluble, it has hydrophilic character which causes it to swell to form particles with gel structure. The degree of swelling is dependent upon the degree of cross-linking, and it is expressed as the water

regain (W_r). Water regain is defined as the number of grams of water imbibed per gram of dry sephadex. The ability of a swollen sephadex gel to exclude molecules exceeding a certain size and to be penetrable by smaller molecules is the basis of the gel filtration procedure.

Sephadex swells in strongly polar solvents as well as in water to form an insoluble gel. The volume of bound solvent per gram of dry substance is a function of the degree of cross-linking of the dextran, which in turn is related to the number of pore sizes in the solute molecules. An aqueous gel column prepared from a gram of dry sephadex contains interstitial water V_o and water in the gel particles V_i, corresponding to a.W_r, where W_r is the water bound (water regain) per gram of dry material, and a is the weight of dry sephadex used to pack such a column. If we consider a solution containing a polysaccharide of high molecular weight and a monosaccharide being added to a column and eluted at a rate such that equilibrium is established throughout the column; then if we assume that all water in the gel, V_i, is available for diffusion of small molecules without being affected by any other factor, such as adsorption, then the monosaccharide, freely diffusing in the interstitial and bound water, will be received throughout the column in volume that is equal to $V_o + V_i$, where V_o is the void volume and V_i is the imbibed volume. The polysaccharide is completely excluded from diffusion in the gel and can be received in a volume that is equal to the void volume V_o. Any restriction of diffusion in the gel lowers the hold-up in the elution and the material is received from the column at some point in between the monosaccharide and the completely excluded polysaccharide. The elution volume, V_e, of a material may therefore be written:

$$V_e = V_o + K_d V_i$$

where K_d is constant, and it is the distribution coefficient of the substance being diffused.

Practically, it has been found that not all the water in the gel particle is available for diffusion of low-molecular-weight substances. For example D-glucose on sephadex G-25 shows a K_d value of about 0.7 - 0.8 (84, 8, 10, 5) instead of the theoretical unity. Such a case, as the adsorption of the substance on the column material increases the elution volume of that substance. Moreover, the pH of the eluting solvent, and the ionic strength of the charged molecules affects separations.

The general expression for the appearance of a molecular species in the effluent is described in terms of the volume of the external water, and of the internal solvent volume trapped in the swollen particles. The distribution coefficient, K_d, is a parameter of the molecular dimension of the volume fraction of V_i that is available for the solutes. The distribution coefficient is zero for molecules that are completely excluded from the network (5, 8, 10, 7, 1, 14) and attains the value of one for molecules distributed equally between the stationary and the mobile phases. With a homologous series, the molcular dimensions increase with increasing molecular weight. The distribution coefficient has the value between 1 and 0 for the molcular species more or less penetrable into the gel network, and a fractionation according to molecular size is assumed to take place within this molecular weight range. As a result of the cross-linking reaction, there is a wide size distribution of the network mashes, thus explaining the gradual change in K_d values over a rather extended molecular weight range.

The distribution coefficient of a molecular species is clearly a function of the water regain of the dextran gel. The range of the molecular weight to be fractionated is thus a decisive factor in the choice of the gel for a fractionation experiment.

The number average molecular weights (M_n) are determined by reducing end-group analysis using Somogyi's phosphate method (30). The weight-average molecular weights are measured for all fractions before the molecular weight determinations (5).

For the fractionation of oligo- and polysaccharides, one is confronted with the fact that the difference in the distribution coefficients between successive members in a homologous series are small and therefore columns of high efficiency need to be used. For example, a column of at least 3,300 theoretical plates is required to separate two fractions of distribution coefficients of 0.7 and 0.8 for isomaltose and glucose, respectively (1, 2).

Because of small differences in distribution coefficients of oligo- and polysaccharides, long columns have to be used with very low rates even though small particle sizes may be used. However, there is an optimum size for column that should not be exceeded. Thus separations take a relatively long time. In addition to that, smaller amounts of polysaccharides compared with desalting procedure (2, 5) can be separated in each experiment.

Oligosaccharides of the isomaltose series were first used to test the probabilty of fractionation (2, 5). It was very soon realized that only

partial separation was obtainable unless very fine gel particles were used.

However, fractionation was obtained using 100-200 mesh particles; and oligosaccharides of the isomaltose series were isolated using Sephadex G-25 200-400 mesh packed in a 3 X 120 cm. long glass column eluted with distilled water (2, 6). In this particular case a flow rate of 20 ml per hour was used and the time for the experiment was 40 hours (6). The fractions were evaporated and identified by paper chromatography.

Oligosaccharides in the dextran series were separated by the Flodin and Apsberg (7) method. Indiviual oligosaccharides up to hexaose were isolated. They were identified by paper chromatography, molecular weight determination and x-ray diffraction.

Factors Affecting Separation

Sample Volume

Since $V_e = V_o + K_d V_i$, two solutes with distribution coefficients K'_d and K''_d will be received at effluent volumes separated by $(K'_d - K''_d)V_i$ (8, 10, 7, 14, 18, 1). In the case of separating monosaccharides (K_d about 0.8) from large polysaccharide colloids ($K_d = 0$), the upper limit of the sample volume is about 0.8 V_i. Practically, it has been found that simple separations are not complete if the sample volume exceeds half of the theoretical value or about 25% of the total bed volume. For chromatographic separations, the samples are applied in much less volume, usually less than 5% of the total bed volume (8, 10, 7, 14, 18, 1).

Sample Concentration and Viscosity

If the viscosity of the sample solution is low, the concentration seems to have little or no influence on the separation except in so far

as the gel structure may be changed by osmotic effects. The limiting viscosity is about 10 to 11.8 centipoises (8, 10, 84, 1, 14, 7).

Ionic and Adsorption Effects

The effects of ion exclusion and ion exchange manifest themselves in the behavior of charged solutes on sephadex gel column by exclusion of low-molecular weight acidic materials and the adsorption of basic substances (10, 5, 14, 18, 4, 1). These effects are only apparent in the absence of electrolytes and are eliminated or greatly reduced if the gel filtration is accomplished in dilute salts solution (10, 5, 14, 18, 4, 1), such as 0.1 M pyridine accetate or 1.0 M acetic acid. In these cases, the column is equilibrated with the developing solution before application of the sample (8, 10, 5, 14), and, by using volatile salts or acids, a quantitative desalting is possible (5, 8, 10, 14, 1).

Size of Column and Gel Particles

The ratio of height to diameter of a column may vary from 4:1 to 40:1 (8, 10, 5, 14). The higher the ratio the better is the resolution, and for simple desalting a ratio of 4:1 is sufficient (8). Dry-sieved gel material gives better results than unsieved substance. The best column efficiency is obtained by using a small particle size, but a small particle size (8, 10, 5, 14) increases the pressure drop and hence decreases the flow rate (8). As a compromise, 100-200 mesh material proves to be suitable for most purposes.

The pore sizes types G-25, G-50, and G-75 Sephadex gels correspond roughly to an exclusion of materials with molecular weights greater than 3500-4500, 8000-10,000, and 40,000-50,000, respectively (72). The most

recently developed types of sephadex G-100 and G-200, extend this spectrum to about 200,000 (72, 5, 8) for polysaccharides. The swelling of the sephadex and the diffusion rates of solute molecules depend to some extent upon the nature of the solvent (8, 10, 5).

Flow rate

The efficiency of the gel filtration increases as the flow rate decreases (8, 10, 5, 14). However, up to certain limits, the flow rate is of minor importance. Since the rate at which solvent drains from a column of Sephadex decreases with both particle size and degree of cross-linking (5, 8, 10, 14, 18), the use of unrestricted flow from types G-50, and G-75, and G-25 of finer than 200 mesh is frequently suitable (5, 10, 8, 14, 18, 1).

Applications

Nordin reported the successful fractionation of Naegeli amylodextrin, products of alpha-amylolysis of amylose, soluble starch, and oligosaccharides of the maltose series using Sephadex G-75 (11).

Oligosaccharides in the cellulodextrin series have been separated on G-25 (12). The fractionation of low-molecular weight dextrans (12) have been performed on the early types of sephadex (G-25 to G-75). The fractionation of dextran by gel filtration using the whole series of G-Series from G-25 to G-200 inclusive have have been reported by Granath (14).

Sephadex has been applied to carbohydrate solution for purposes of

desalting and fractionation according to molecular weight (10, 13). Fractionation of mucopolysaccharides from ECTEOLA-cellulose chromatography (10, 13) and products from periodate oxidations (15) have been isolated free of the respective salts. D-Glucose and dextran in biological samples have been separated prior to the determination of the dextran (15, 16).

The celludextrins up to cellulohexaose have been separated on 200-400 mesh sephadex G-25 (12), and the course of the alpha-amylolysis of glycogen has been similarly followed (17). Starch dextrins have been partially resolved on sephadex G-75 (11). Dextran has been fractionated according to molecular size (18). Gel filtration has been extensively applied in the fields of proteins (10) and enzymology (19) and has been used to isolate glycopeptides from ovalbumin, and to fractionate amylose acid hydrolysates (11) according to molecular size.

Solubility of Sephadex (22, 23, 14, 2, 8, 10, 18)

It has been found that when sephadex was swollen in water, and after 24 hours when it was washed and resuspended in fresh water, it gave an anthrone reaction in the dried extracts indicating that there is a carbohydrate extracted by distilled water (12, 8). During the first 24 hours, in an experiment, 0.1 - 1% of the gel was extracted. However, the amount varies with the batch and the type of gel (8, 9, 10, 11, 12). After 4-5 days constant values were obtained and the release of soluble carbohydrate material was 0.002 - 0.003% per day. For example, sephadex G-25 gave a constant release of soluble material obtained after four days amounting to 0.01% per day. At 65°C higher values were obtained.

Comparision Between Types of Sephadex (22,23,14,2,8,10,18)

1. Solubility: The higher G-Series such as G-200, and G-100 are much more water soluble when compared to lower G-Series such as G-75, G-50, G-25, etc. This is because the dextran to start with is water soluble, made insoluble by cross linking with epichlorohydrin, and the degree of cross linking is less with the higher numbers (8).
2. The Fines: These are insoluble particles of smaller size than the remainder of the sample. However, these materials are at higher concentration in the lower G-Series when compared to higher G-Series (8-12).
3. Column Preparation: In the lower G-Series, it is necessary to decant and wash several times to eliminate fines. Moreover, the column after packing should be washed several times until the background becomes constant. After that it will remain constant (8 - 14). While in the higher G-Series, there is less fines to begin with (22, 23, 14, 14, 2, 8), after the column has been prepared more soluble carbohydrates will come out of the column because of less cross-linking.

Methods for D.P. Determination

Most of the techniques described depend upon chemical reaction with the reducing group of the carbohydrate in question. This reaction must produce products which can be quantitatively measured. The extent of the reaction will be inversely related to D.P.

The end group in carbohydrate is a reducing group which can be determined by various oxidizing reagents such as some selected copper salts.

Certain alkaline copper reagents have been used extensively(24, 25)

for end group analysis. The literature that is available about copper as an oxidizing agent for the carbonyl group of carbohydrates is so large that is appears to be one of the most important cations used for the end group group analysis (24, 25, 26, 27).

The copper reduction methods are either gravimetric, the CuO that is formed is weighed, or volumetric. The latter is usually conducted by adding H_2SO_4 and KI. The iodine that is produced, being an equivalent amount of CuO is titrated generally by sodium thiosulfate in the presence of starch indicator (24 - 27).

Somogyi Micro Copper Method

It is possible that this method is one of the most widely used methods. The method in which alkaline copper is used and which was developed over many years by Shaffer, Hartmann, and Somogyi is one of the most widely used methods for D.P. determinations of Oligo- and Polysaccharides (28, 30). The copper cation is more specific for sugars (29, 30, 31, 32) rather than hypoiodite or ferricyanide (29, 30, 31, 32); and therefore, it is preferred in analysis of biological materials.

It should be noted that the usage of sodium sulfates (30, 33) has considerably reduced the back-oxidation by air; however the air-oxidation is still a handicap that should be prevented. At the same time autoreduction of the alkaline tartrate copper reagent during heating is another difficulty of the method (28, 34, 36).

However, the Somogyi's method has been applied successfully on both the milligram and microgram scales using both the titrimetric and colorimetric procedures. Moreover, it should be noted that the method is precise

over a wide range of sugar concentrations; and that it is easy and rapid to operate with highly reliable and reproducible results. All this has placed the method above other micro oxidation methods (36,28,37,38,39,32 35).

The titrimetric and colorimetric modifications of the method are recommended for the determination of unsubstituted reducing sugars resulting from chromatographic separations. The copper cation is available in different compositions; some of which uses Nelson's arsenomolybdate colorimetric reagent or the iodimetric titration procedure (40, 41, 42).

Nelson's method is a modification of Somogyi's and it is as precise as the titrimetric method. This method gives reliable results in the range from 5 - 600 gamma; and this is a wider range than usual for colorimetric methods. Nelson's method should be used only when the copper-oxidized sugar solution is transparent and relatively colorless. His reagent determined the reduced copper reacting it with phospho- or arsenomolybdate. Those reagents are color-forming salts. The arsenomolybdate method produces a more stable color and is more reproducible. Nelson's modification of Somogyi's has been used with both Somogyi 1937 and 1945 reagents (43, 30, 38, 34, 36). However, according to Somogyi (28), both the 1937 and 1945 reagents are poorly composed for use with Nelson's modification, and a different composition is now used (28).

Wager reported that 1945 reagent is satisfactory, but only if it was possible to exclude air during mixing, heating and cooling (44). Wager also pointed out that a low-alkalinity carbonate-hydrogen carbonate reagent is suitable for use with Nelson's colorimetric method. This reagent is similar to Somogyi's latest recommendation.

On the other hand, Paleg reported that D-glucose, D-fructose, and maltose gave reproducible results in accordance with Somogyi's latest recommendation, but the presence of citrate buffer in the sugar solution depresses the absorbance values within the range 500 - 650 mu (45).

It should be noted that polysaccharides (46) containing carbonyl groups undergo depolymerization and oxidation by dissolved oxygen in alkali, and are otherwise more reactive therein than in acids (47, 48, 49). Most of the analytical reagents for carbonyl in sugars, such as hypiodite, copper, and ferricyanide, require alkali for reaction, and have been applied, often without qualification to polysaccharides.

Many of the alkaline copper reagents have been recommended for use in determination of reduced copper iodimetrically. Somogyi's 1945 phosphate-buffered reagent is preferred over 1952 carbonate-buffered reagent, and that is because amyloses are held in solution in the 1945 reagent while they are precipitated from the 1952 reagent (44, 50, 28).

The 1945 reagent has been used to determine the molecular weights of oligo- and polysaccharides (51, 52). It has been observed that the sugar equivalents of the 1945 reagent will vary from operator to operator and from time to time (34, 35, 53, 54); and therefore, sugars standards should be run frequently and it is preferred that they are done with each group of analysis. One advantage of the 1952 reagent is that its sugar equivalents remain unchanged for several years (28).

This method is precise within $\pm$ 0.01 mg. for maltose and D-glucose. This also means that it is precise within $\pm$ 2% average in the range from 0.3 to 3.0 mg. of glucose. It may be mentioned that with this method as

little as 5 gamma of sugar could be determined in a plant tissue with a precision of ± 5% (35). The most reproducible results could be obtained with this method, when air is eliminated from the copper reagent, and when mixing and heating procedures are carried out under an inert atmosphere (34). It is better to preheat the alkaline copper reagent before the addition of iodate in order to get the greatest precision in the microgram range (35).

The presence of alkali makes the general applicability of such methods to aldehyde measurements of polysaccharides questionable (46).

Copper reacts nonstoichiometrically with carbonyls, yielding varying copper-sugar equivalents: 9.2 for cellobiose for example (55). In spite of this, copper reagents have often been applied to polysaccharides as in measurements of D.P. of native and clinical dextrans (56), of hydrolyzed dextrans, of starch (57), and in measurements of "reducing power" of cotton cellulose, hydrocellulose, periodate cellulose, periodate dextran, and periodate cornstarch.

Stewart and Nordin (58) developed a method to determine the degree of polymerization of Oligosaccharides. They used the alkaline copper phosphate reagent known as Somogyi 1945 reagent. Total carbohydrate was analyzed by the anthrone method (59), and the degree of polymerization of oligosaccharides was determined from the ratio of total glucose by anthrone method to glucose equivalent by Somogyi method (30).

$$\text{D.P.} = \frac{\text{Total glucose by anthrone method}}{\text{Glucose equivalent by Somogyi method}}$$

This method gave good results for the determination of degree of polymeriza-

tion of maltooligosacchrides from G_1 to G_6 and it was proposed that the method could be applied to dextrins provided that a 20 minute heating period was used.

Also Launer and Tomimatsu (46) described a method for the determination of degree of polymerization of polysaccharides. They used different types of carbohydrates, including amylose, amylopectin, native and hydrolyzed dextrans, periodate corn starches, polygalacturonide, and Araban. Also they used different alkaline methods including Somogyi's 1945 and 1952 alkaline copper reagents, ferricyanide alkaline reagents at different molarities (46), and hypoiodite at different molarities. And for the comparision they used chlorite method before and after the addition of alkali. The D.P.'s were determined from the following formula:

$$D.P. = \frac{E \times W}{V \times N \times M}$$

where

E = number of equivalent of oxidizing agent corresponding to 180 grams of glucose (46).

W = milligrams of substance per aliquot.

V = milliliters of thiosulfate used.

N = normality of thiosulfate used.

M = Formula weight of AGU (46).

They found that different alkaline methods give different results, and that the alkaline medium which is necessary for reaction will affect the aldehydic group whatever the method may be, chlorite, ferricyanide, or Somogyi's, and that the alkalinity of copper reagent will destroy the aldehydic groups without copper reduction (46).

The need for observing precautions to retard air oxidation is then apparent when alkaline copper reagents are used. Thus a good practice is to use two or more methods for comparative purposes.

II. Iodine Stain Technique for D.P. Determination

Bailey and Whelan (60) developed the iodine stain technique for the determination of the Degree of Polymerization of starch polymers or dextrins. Synthetic amyloses of known D.P.'s were prepared by the reaction of phophorylase with glucose-1-phosphate.

The iodine staining properties of the synthetic amyloses were measured by iodine solution. The light absorption over the range 450 to 700 mu was measured using 1 cm cells.

The iodine solution used to stain the synthetic amylose contained 0.2% iodine in 2% potassium iodide as is used in the routine determination of the "blue value" (61). The concentration of amylose was calculated from the initial amount of maltohexaose and the inorganic phosphate released. That is to say, the Degree of Polymerization of the amylose was calculated from the amount of inorganic phosphate released (and hence the moles of D-glucose incorporated into the polymer) during synthesis from a known amount of pure maltodextrin primer (63). Mould and Synge found that the glucose units were added in random fashion to the end of each (64) priming molecules so that the resulting polymer had Poisson-type distribution of chain length which could be calculated accurately (64).

Since phosphorylase-catalysed synthesis of amylose was shown (63) to involve the completely random addition of glucose units to all available priming ends (multichain synthesis), the resulting polymer which was built

up was one in which the various molecules were present in a Poisson distribution (64). The form of this distribution may be calculated from the formula:

$$P_x = \frac{e^{-v} \cdot v^x}{x!}$$

where P_x is the fraction of the priming molecules which have had x glucose units added, when v is the average number of glucose units added per priming molecule. A series of different D.P.'s were calculated by the Poisson formula given above. Those values were tabulated versus the corresponding maximum absorbance measured. Thus an emperical method for D.P. determination of amyloses is available.

When the iodine stain for an amylose sample is measured in the manner described, the tables can be used to determine the D.P. of amylose by reading the corresponding value of the maximum absorbance from the table.

The iodine stain can be conducted without purification of the sample (60) because buffer salts, alpha-D-Glucose-1-phosphate, etc. do not interfere (60). Also it has been found that only the values of wave length of maximum absorbance are needed. Using this emperical method the minimal D.P. necessary for iodine-staining under the standard condition is 18 units (60). That is to say 18 glucose units are the minimum necessary for formation of an iodine complex have a visible stain.

Varying the amounts of alpha-D-Glucose-1-phosphate and maltohexaose will result in variation of values for the same wave lengths, and the average of the two values should be considered (60). For example at 610 mu one table will have a value of 81, and the other will have 107 and the average is 94. That is to say, the maximum absorbance at 610 mu or higher is not

linear with the D.P.'s calculated from Poisson distribution formula. For example the absorbance will remain constant, while the D.P.'s will increase with no increase in the corresponding maximum absorbance. The absorbance will remain at 620 mu while the D.P.'s will change gradually from 169 to 218 (60). At higher maximum absorbance such as 645 mu, this is more obvious; where the absorbance will remain constant at 645 while the D.P.'s will change from 325 to 568 with no change in the corresponding absorbance. Thus the application of this method to D.P.'s higher than 70 is questionable.

The formation of color by the interaction of starch and iodine is one of the most useful and characteristic reactions of the polysaccharide. The relatively advanced state of our knowledge of starch structure is due in great measure to the ability of iodine to detect small amount of starch and to reveal changes in its degree of polymerization caused by enzymatic and chemical treatment. The blue color of stain is due to the amylose component of starch. The other component, amylopectin, gives a red-purple color which is much less intense than the amylose stain. When hydrolyzed in random fashion by acid or by alpha-amylase, both polysaccharides gradually lose the capacity to stain with iodine. The amylose color becomes purple, then red, brown, and finally disappears.

Rundle and Co-workers (65) have proposed a helical, molecular structure for amylose iodine complex. The entering of iodine molecules are presumed to be polarized by the permanent dipole moment of the amylose molecule, and they in turn interact thru dipolar forces. There is evidence to suggest that the larger the amylose molecule, the greater the iodine affinity and the larger the wave length of absorption.

Meyer and Bernfeld (66) have rejected the helical theory and have

presented some evidence that amylose micelles are essential for complex formation. There are excellent articles (67, 68, 69, 70) which discusse the iodine stain complex of the amylose and amylopectin. Since the color of iodine stained amylose is blue and that of amylopectin is purple to red, it seemed likely that a difference in ability of amylose and amylopectin to bind iodine in complex formation existed.

The nature of the iodine amylose complexes was first to be developed from the preliminary observation on the decrease in the iodine color of starch during enzymatic degradation. A coil of 6 glucose units was necessary for the display of iodine staining properties, and that the amylose chain was coiled in solution, giving a long spiral. Each turn of this spiral contained 6 glucose units, and the iodine molecules lay in the interior. Moreover, the amylose molecule could exist in the form of a helical spiral with each turn of the helix containing 6 glucose units, and that the interior of the helix had a "hydrocarbon lining" due to the inward-pointing hydrogen atoms of the glucose units. The stain given with iodine was attributed to this lining, by analogy with the purple color potentiometric technique (71) it was shown that neutral amylose bind iodine strongly, and in amounts up to 21% by weight, corresponding to 6 glucose units for each iodine atom. X-ray diffraction studies were in agreement with the helical structure, with the iodine molecules arranged the spiral with their axes along its major axis.

The complex formed between amylose and iodine in dilute determination of blue value, was shown to be developed when the ions of the type

$3I_2$ $2I^-$ were present. Such ions are probably present in a linear resonating form, and that the red complexes which were formed by shorter chains were due to their inability to stabilize an ion of this size.

One of the most important factors determing iodine stain in the low molecular weight region is the iodine concentration. It may be added that certain polysaccharides such as amylose, amylopectin, glycogen, and dextrin form characteristic color complexes when combined with molecular iodine (65 - 71). An absorptive complex between iodine and helically coiled polysaccharide chains takes place. Chain length of at least 6-8 linear sugar residues are required for the ready formation of helixes and intense iodine complexes. Thus linear or helically coiled polysaccharides, such as amylose and starches which are rich in amylose, form an intense blue-black color with iodine. Branched polysaccharides with interrupted helixes, for example amylopectin, yield less intensly colored iodine complex; and highly branched polysaccharides with short segments and hindered helix formation, for example glycogen, yield only pale brown color complexes with iodine.

However, the reaction of starch with iodine giving a blue color complex, may be summarized as follows (65 - 71):

1. The intensity of color depends on the ratio of amylose to amylopectin in a given sample of starch, the iodine with amylose gives a blue color, and a purple color with amylopectin.

2. Starch saturated with iodine binds one iodine molecule per 6-8 glucose units. Oligosaccharides of short chain length bind no iodine.

3. Iodine stain colors are not produced unless the medium is aqueous

and in the presence of pot. iodide. For example, it tests negative when iodine solutions are used without iodide salts, or when iodine is dissolved in chloroform instead of water.

4. In order for the test to be positive, the medium should be acidic or neutral. The test is negative in alkaline solutions, in methyl alcohol, and in absolute alcohol.

5. The medium should be free of inhibitors such as chloralhydrate, resorcinol, pyrogallol, sodium hydroxide, and fatty acids.

6. The blue color disappears by heating and then reappears by cooling. The intensity of the blue color is proportional with the temperature required for its disappearance, and this is in the iodine starch complex. This is explained on the basis that the iodine is absorbed on the glucose molecules in between the starch coils; and that is because of the helical structure of starch. The heating will cause the stretch of the coils and or the helix, and this causes the iodine to stay away from those stretched coils. However, upon cooling the starch coils return to their normal size, and iodine which was staying away will be reabsorbed by the coils and the blue color will appear again (65-71).

7. It has been observed that salts such as Na_2SO_4, NaCl, $CaCl_2$, and KI when they are available in large quantities cause the starch iodine complex to precipitate. This precipitate has been observed by several authors (65-71) to contain variations in its iodine content from 2-23%. In general the amount of iodine in the complex is proportional to amount of iodine used and also the presence of other chemicals in the medium.

8. The iodine test will be positive with oxidized amylose up to 80% of oxidation. If the amylose is oxidized more than that the iodine

test is negative. Also the iodine test is positive with methylated and acetylated starch. This indicates that the OH-groups do not do much in the formation of the blue complex.

9. In the absence of inhibitors, it has been found that 0.15 ml of 0.001 N of iodine solution in 50 parts of KI can detect 1 part of starch which was dissolved in 50,000 parts of distilled water.

10. Mechanism of starch iodine reaction

a. The iodine is absorbed on the starch. However, it has been observed (65 - 71) that compounds which can form oxygen bridge such as cyclo-acetyl acetone gives a blue complex with iodine.

b. The starch easily forms a helical structure, and a coil will have a turn containing 6-8 glucose units wherein the iodine molecule can settle to give the blue color.

c. The blue color can not be produced with amylopectin, and that is because of the absence of the coil and helical structure. Moreover, the glucose units should be in the pyranose form in order to give the blue color.

d. The density of the iodine color produced is proportional to the chain length (60, 65) and it was found that amylose samples can absorb 19% of their weight in iodine.

MATERIALS AND METHODS

I. Partial Acid Hydrolysis of Amylose

Two samples were prepared, and each was prepared in a slightly different manner. They are referred to as Sample No.I and No.II.

Sampel No.I. 20 grams of Commercial amylose (Stein Hall Inc., Superlose) was dissolved in 200 ml of dimethyl sulfoxide $(CH_3)_2SO$. It was allowed to stand overnight, then the dissolved amylose was crystallized (precipitated) by the addition of 1-butanol. After 24 hours, the supernatant was decanted and the precipitated amylose-butanol complex was air dried. The air dried sample was hydrolyzed with 250 ml of 0.01 N HCL under reflux. Aliquots were withdrawn at 10 minutes, and cooled immediately by immersing in an ice bath. They were neutralized with 1 N ammonium hydroxide and dried under vacuum.

Sample No.II. 20 grams of amylose were dissolved in 150 ml of dimethyl sulfoxide (3-4 days). Then amylose was precipitated by 1-butanol, the supernatant was decanted and the moistened sample was partially hydrolyzed using 200 ml of 0.01 N HCl. Aliquots of 10 and 20 minutes were withdrawn and cooled immediately in an ice bath. After neutralization with 1 N ammonium hydroxide, the samples were lyophilized. They were designated as Sample No.2-10 and Sample No.2-20. Each hydrolysate was fractionated through a sephadex column of G-200 or G-100.

II. Gel Filtration

The gel filtration was performed on a column of Sephadex G-200 or

G-100, 140-400 mesh (Pharmacia, Rochester, Minnesota). The column size was 61 x 1.5 cm (bed volume of 120 ml) in case of G-100, and 71 x 1.7 cm (bed volum of 140 ml) in case of G-200.

Sephadex was first soaked in distilled water for 3 days (72) in order to obtained maximum swelling of the gel bead. The excess of water and fine materials were decanted. The residue was poured carefully into the sephadex column which contained about one third its volume of distilled water to avoid entrapped air bubbles and to produce uniform packing. Then it was washed with water at least 3 times the bed volume before the application of the sample. 100 mg samples of amylose hydrolysate was dissolved in 1 ml of DMSO and diluted to 25 ml with water. 5 ml of this aqueous solution containing 20 mg of sample hydrolysate was injected into the column, and eluted using distilled water. 5 ml fractions were collected using a fraction collector. Collected fractions were received in acid cleaned test tubes; and stored over a few drops of chloroform (11) with refrigation until ready for analysis. The chloroform forms a complex with amylose which will dissolve by warming, releasing the volatile solvent (11).

III. Total Carbohydrate Determinations:

The total carbohydrate content of fractionated hydrolysates was determined using the phenol sulfuric acid method (73, 74). To a final of diluted carbohydrate solution of 1 ml; 1 ml of 5 % phenol in distilled water was added and mixed. This was followed by the addition of 5 ml of 96% H_2SO_4. Each tube was agitated during the acid addition and the same mixing procedure was used each time. After 10 minutes the tubes were reshaken and placed in a water bath at 25°-30°C for 20 minutes. The ab-

sorbance of the yellow orange color was measured at 490 mu using a maltose standard. A linear response was obtained with the method up to 70 gamma per ml. Measurements were done with Bausch & Lomb Aloe Spectrophotometer.

IV. Determination of Degree of Polymerization by Iodine Staining:

The relation between the iodine stain and chain length has been reported by J. M. Bailey and W. J. Whelan (60). To 2 ml of sample was added 1 ml of 1 N NaOH followed by heating in a boiling water bath for exactly 3 minutes. It was then cooled immediately in running tap water, followed by the addition of 1 ml 1 N HCl for neutralization. Potassium hydrogen tartrate (0.07 - 0.05 g.) was added to the volumetric flask. Then the volumetric flask was filled to 22.5 ml with distilled water; before the addition of 0.25 ml of iodine solution (0.2% in 2% KI). The flasks were then filled to 25 ml, shaken and left to stand for twenty minutes at room temperture. The maximum absorbance was measured using a Beckman DB or DU Recording Spectrophotometer, in the range from 760 mu to 320 mu against a reagent blank.

V. Determination of Degree of Polymerization by Nelson's Copper Reagent(43):

Nelson's alkaline copper reagent was prepared (43, 75); and 1 ml of this reagent is added to 3 ml carbohydrate aliquots. This was done in a 25 ml Folin tubes, with marbles placed in the mouths of the tubes. The tubes were heated for exactly 20 minutes in a boiling water bath, and then cooled simultaneously to 25°C. To each tube was added 1 ml of arsenomolybdate reagent, and the tubes were shaken occasionally during 5 minutes to dissolve the precipitated Cu_2O and to reduce the arsenomolybdate. Then tube contents were diluted to 10 ml with distilled water, and absorbance was taken at 540 mu in 1 cm. cell against reagent blank. The amount of carbohydrates were calculated from maltose standard using 200 ug/ml. Measurements were done with Bausch & Lomb Aloe Spectrophotometer.

RESULTS

Results obtained using the phenol sulfuric acid method (73, 74) are presented in Table I which is accompanied by the plot of the total carbohyrate content versus the fraction number (Figure I). This figure shows the elution pattern of the Sample No.1-10 using Sephadex G-100.

Elution diagrams using Sephadex G-200 with the phenol sulfuric acid method were not reproducible and the background could not be controlled. Therefore, they are not shown. Nelson's method (43) gave good results and D.P.'s determined by this method and by Iodine Stain Technique (63) are shown in Table I.

Results for the iodine staining technique for the determination of the D.P.'s are shown in Tables I through IV inclusive.

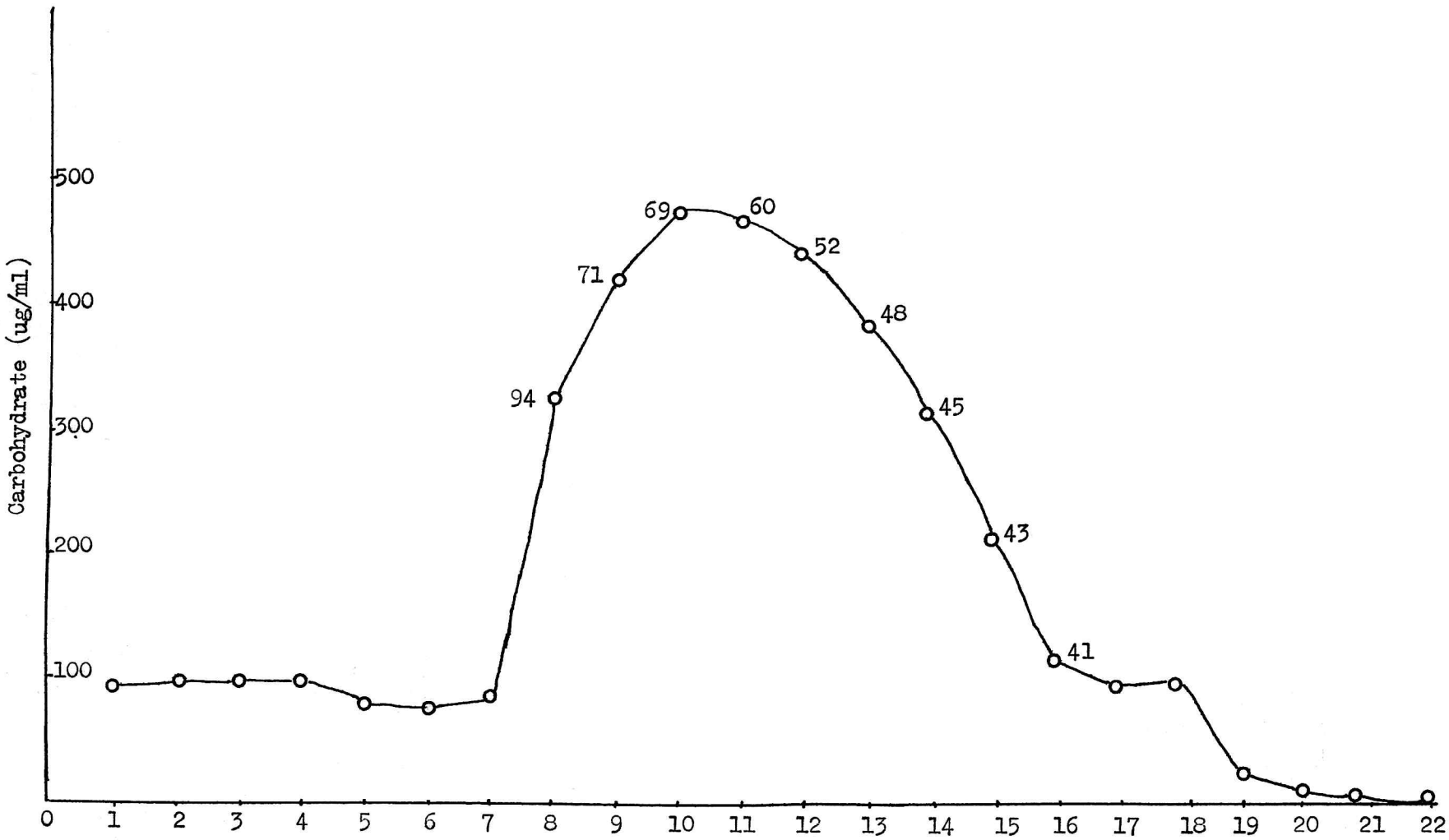
Table II shows the iodine absorption data obtained for the first Sample No.1-10 using G-200 where the table shows the wavelength at which the maximum absorbance was obtained, the corresponding D.P.'s and the colors observed.

Tables III and IV show the iodine absorption data obtained for the second Sample No.2-10 and No.2-20. They show the wavelength of maximum absorbance, the D.P.'s obtained and the colors fractionated.

Figures II and III show colored pictures for the fractionated samples using Sample No.1-10 minutes hydrolysate on Sephadex G-100 and G-200 respectively. In all cases, each table or figure is accompanied by an explanation statement on separate page.

Explanation of Figure (1)

Figure (1) - Shows the elution diagram for Sample No.1-10 minutes hydrolysate, showing the total carbohydrate analysis using the Phenol Sulfuric Acid Technique. Sample has been fractionated using G-100. 5 ml sample containg 20 mg. has been fractionated, and recovery obtained was 100%. (See Figure 2 for the fractionated colors).


Carbohydrate (ug/ml)
500
400
300
200
100
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
94
71
69
60
52
48
45
43
41

Explanation of Figure (2)

Figure (2) - Shows a colored picture for the fractionated sample No.1-10 minutes hydrolysate. 20 mg. sample was used and recovery was 100% (See Figure (1) and its explanation). The picture shows colors obtained for fractions No.8 thru No.16 inclusive, with the maximum absorbance determined for each fraction in mu units, and under which is the D.P.'s as determined from the Iodine Stain Technique(60). The fractionation has been done on G-100.

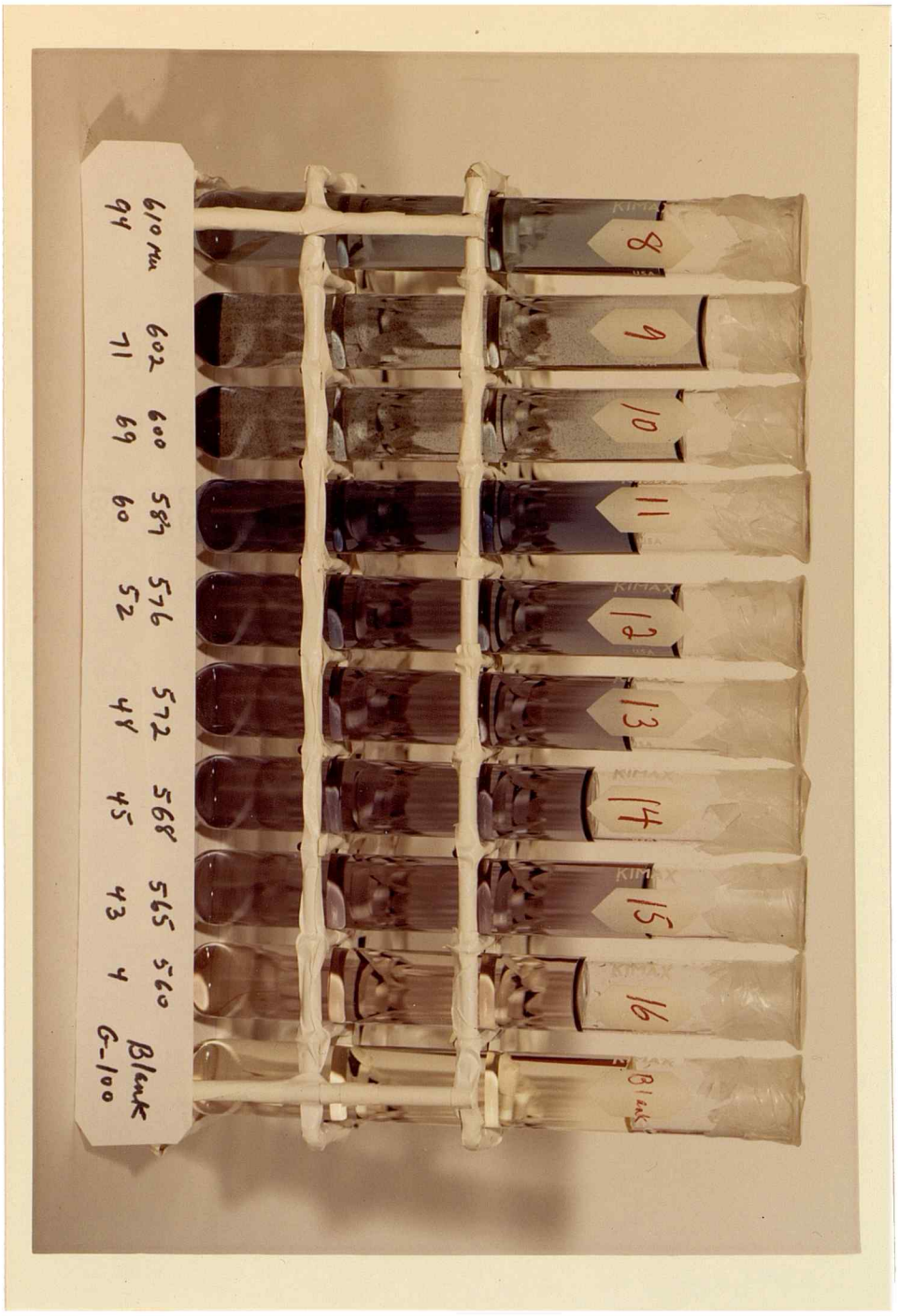

8
9
10
11
12
13
14
15
16
Blank
6/10 mu 602 600 589 576 572 568 565 560
94 71 69 60 52 48 45 43 4
G-100

FIGURE II

Explanation of Figure (3)

Figure (3) - Shows a colored picture for the fractionated Sample No.1-10 minutes hydrolysate. 20 mg. sample was used and was fractionated using G-200. The picture shows colors obtained for fractions No.16 thru No.25 inclusive (See table II), with the maximum absorbace determined for each fraction in mu units, and under which is the D.P.'s as determined from the iodine stain Technique (60).


16
17
18
19
20
21
22
23
24
605mu 596 580 596 584 588 564 576 560 552
83 62 55 62 56 56 43 49 41 36
G-200

FIGURE III

Explanation of Table I

Table I - shows the D.P. determinations using Nelson's and Iodine Stain Methods. 50 mg. sample was fractionated, and D.P.'s using Iodine Stain Technique were determined for those fractions which gave positive iodine stain colors (fractions No.7 thru No.17 inclusive). Measurement of D.P. by Nelson's alkaline copper reagent are also shown for the same fractions.

Table I

D.P. Determination by Nelson's and Iodine Stain Methods
Using G-100 and Sample No.1-10.

(1) Tube Number (5 ml)	(2) Total CHO from Phenol-H_2SO_4 (ug/ml)	(3) Total Maltose from Nelson's (ug/ml)	D.P. Nelson's = $\frac{(2)}{(3)} \times 2$	D.P. Iodine Stain
1	17.50	---	---	---
2	14.00	---	---	---
3	28.00	---	---	---
4	17.50	---	---	---
5	70.00	---	---	---
6	231.00	---	---	---
7	637.00	13.60	93.80	94.00
8	882.00	21.30	82.80	83.00
9	896.00	25.30	70.80	71.00
10	896.00	26.00	68.80	69.00
11	861.00	24.60	70.00	69.00
12	798.00	26.00	61.40	62.00
13	763.00	25.00	61.00	61.00
14	673.00	21.00	60.60	61.00
15	588.00	19.50	60.00	60.00
16	459.00	18.30	50.00	50.00
17	315.00	15.60	40.20	40.00
18	245.00	---	---	---
19	179.00	---	---	---
20	123.00	---	---	---
21	74.00	---	---	---
22	74.00	---	---	---
23	17.50	---	---	---
24	7.00	---	---	---
25	7.00	---	---	---
26	0.00	---	---	---

Explanation of Table II

Table II - Shows the Iodine Absorption Data for sample No.1-10 minutes hydrolysate. Sample has been fractionated according to colors and to molecular size (See Figure (3)). Table shows, as well, the wave length of maximum absorbance, and the D.P.'s as determined by the iodine stain technique. Sample has been fractionated thru G-200.

Table II

Iodine Absorption Data Using G-200
Sample No.1-10

Tube Number	λ max mu	D.P. from Iodine stain technique	Molecular Weight	Iodine Stain Color obtained
1	350	---	---	yellow
2	352	---	---	yellow
3	352	---	---	yellow
4	350	---	---	yellow
5	352	---	---	yellow
6	352	---	---	yellow
7	352	---	---	yellow
8	352	---	---	yellow
9	352	---	---	yellow
10	352	---	---	yellow
11	566	---	---	yellow green
12	572	---	---	yellow green
13	570	---	---	pale green
14	610	94.00	15,224	blue green
15	608	89.50	14,499	blue
16	605	82.50	13,365	blue
17	596	61.90	10,028	blue
18	580	54.60	8,845	blue
19	596	61.90	10,028	blue
20	588	55.75	9,032	blue
21	588	55.75	9,032	blue
22	564	42.70	6,917	purple
23	576	48.60	7,873	blue purple
24	560	39.60	6,415	purple red
25	552	36.20	5,864	purple red
26	530	30.00	4,860	pale purple
27	540	31.60	5,119	red
28	350	---	---	yellow
29	350	---	---	yellow
30	350	---	---	yellow
31	350	---	---	yellow
32	350	---	---	yellow
33	350	---	---	yellow
34	350	---	---	yellow
35	350	---	---	yellow
36	350	---	---	yellow
37	350	---	---	yellow
38	350	---	---	yellow
39	350	---	---	yellow
40	350	---	---	yellow

Explanation of Table III

Table III - Shows the Iodine Absorption Data for Sample No.2-10 minutes hydrolysate. Sample has been fractionated according to colors and to molecular size. Table shows, as well, the wave length of maximum absorbance, and the D.P.'s as determined by the iodine stain technique. Sample has been fractionated thru G-200.

Table III

Iodine Absorption Date Using G-200
Sample No.2-10

Tube Number	λ max mu	D.P. from Iodine Stain Technique	Molecular Weight	Iodine Stain Color Obtained
1	350	---	---	yellow
2	350	---	---	yellow
3	350	---	---	yellow
4	350	---	---	yellow
5	350	---	---	yellow
6	350	---	---	yellow
7	350	---	---	yellow
8	350	---	---	yellow
9	350	---	---	yellow
10	350	---	---	yellow
11	350	---	---	yellow
12	350	---	---	yellow
13	350	---	---	yellow
14	350	---	---	yellow
15	350	---	---	yellow
16	580	---	---	pale yellow g
17	---	---	---	-----
18	596	61.90	10,028	blue green
19	592	59.05	9,566	blue green
20	590	58.25	9,437	blue green
21	592	59.05	9,566	blue
22	588	55.72	9,032	blue
23	584	55.05	8,918	blue
24	580	54.60	8,845	blue
25	568	44.90	7,274	purple blue
26	564	42.70	7,906	purple
27	560	41.04	6,648	purple
28	568	44.40	7,193	pale purple
29	560	41.04	6,648	pale red
30	350	---	---	yellow
31	350	---	---	yellow
32	350	---	---	yellow
33	350	---	---	yellow
34	350	---	---	yellow
35	350	---	---	yellow
36	350	---	---	yellow
37	350	---	---	yellow
38	350	---	---	yellow
39	350	---	---	yellow
40	350	---	---	yellow

Explanation of Table IV

Table V - Shows the Iodine Absorption Data for Sample No.2-20 minutes hydrolysate. Sample has been fractionated according to colors and to molcular size. Talbe shows, as well, the wave lengths of the maximum absorbance, and the D.P.'s as determined by the iodine stain technique. Sample has been fractionated thru G-200.

Table IV

Iodine Absorption Data Using G-200
Sample No.2-20

Tube Number	λ max mu	D.P. from Iodine Stain Technique	Molecular Weight	Iodine Stain Color Obtained
1	350	---	---	yellow
2	350	---	---	yellow
3	350	---	---	yellow
4	350	---	---	yellow
5	350	---	---	yellow
6	350	---	---	yellow
7	350	---	---	yellow
8	350	---	---	yellow
9	350	---	---	yellow
10	350	---	---	yellow
11	350	---	---	yellow
12	350	---	---	yellow
13	350	---	---	yellow
14	350	---	---	yellow
15	350	---	---	yellow
16	350	---	---	yellow
17	350	---	---	yellow
18	350	---	---	yellow
19	588	57.19	9,280	blue green
20	560	41.04	6,648	blue green
21	580	54.25	8,789	blue
22	592	59.05	9,566	blue
23	588	57.29	9,280	blue
24	588	57.29	9,280	blue
25	560	41.04	6,648	blue
26	576	51.13	8,283	blue
27	566	43.77	7,091	blue
28	560	41.04	6,648	blue
29	556	38.97	6,312	purple
30	560	41.04	6,648	blue
31	350	---	---	yellow
32	350	---	---	yellow
33	350	---	---	yellow
33	350	---	---	yellow
34	350	---	---	yellow
35	350	---	---	yellow
36	350	---	---	yellow
37	350	---	---	yellow
38	350	---	---	yellow
39	350	---	---	yellow
40	350	---	---	yellow

DISCUSIONS

I. Total Carbohydrate Determination

The phenol-sulfuric acid method, developed and first applied at the University of Minnesota (75, 76) is simple, rapid, sensitive, accurate, specific for carbohydrates, and widely applicable. It is an excellent method for determination of sugars eluted from spots on paper chromatograms. The reagents are inexpensive, readily available, and stable. A heating bath is not required, stable color is produced, and results are reproducible. It was found that in common with the anthrone method, over which the Minnesota method has several advantages, careful attention must be given to the exclusion of cellulosic lint and filter paper fibers. Under proper conditions, the phenol-sulfuric method is accurate to $\pm$ 2% (76). In the direct determination of lactose in milk and cheese, casein, amino acids, lactic acid, and citric acid in the amounts found in cheese do not interfere with the phenol-sulfuric acid method (77).

Absorbances are measured at 490 mu for hexoses, uronic acids, and their methylated derivatives. The average absorbance of the blanks is subtracted, and the amount of carbohydrate is determined by refrence to a standard curve previously prepared for the particular carbohydrate being assayed. Determinations in triplicates (73, 74) minimize errors and allow exclusion of gross anomalies resulting from accidental contamination with lint and paper fibers.

Total carbohydrate analysis using the phenol-sulfuric acid technique

was carried out for all the collected fractions, in order to construct the elution diagrams. When total carbohydrate content for each of the fractions was ploted versus the fraction number, a continuous curve was obtained resembling a normal distribution, but a small shoulder to the right where oligosaccharides constitute the main components of this part of the curve.

The highest carbohydrate content obtained was at fraction No.10; and as seen from Figure I, the Sample No.1-10 was fractionated into a continuous curve, with gradual increase in the total carbohydrate content beginning from fraction No.7 and reaching a maximum at fraction No.10 after which the total carbohydrate content of each fraction decreases gradually until they vanished at fraction No.20

Further discusions regarding Figure I will be done on the section on D.P. determinations using iodine stain technique (60, 61). and Separations with G-100 (14).

D.P. Determinations

I. Iodine Stain Technique (60, 61).

This is a good technique to determine the Degree of Polymerization of polysaccharides, however, the limitation of this method is that

1. It is insensitive at higher wavelengths of maximum absorbance such as 610 mu, where the D.P.'s average to about 94. It should be noted that at higher wave lengths such as 620, 630, and 645 mu the method become completely insensitive (60, 61). For example, at 645 mu, the D.P.'s will change from 366 to 568 with no change in the measured maximum absorbance at all (60). It should be noted that in this

work no maximum absorbance was measured above 610 mu, so all measurements were within the sensitive region of the method (60).

2. That the iodine concentration is an important factor in the low molecular weight region (60), and for example, the "blue value" (61) of a maltodextrin fraction of average D.P. 24 was increased 50% in intensity by a 10-fold increase in iodine concentration over the standard concentration used by the method (60). Thus the iodine concentration was carefully controlled.
3. The degree of polymerization is another factor in the low molecular weight region. For example, when the above maltodextrin of average D.P. 24 was hydrolyzed by acid to about half its original size, the "blue value" (61) fell to a small fraction of its original value.
4. It also has been observed that in the lower molecular weight region, the maximum absorbance, under the standard conditions of the method, of the measured carbohydrate is not sharp; and a flat curve is obtained most of the time.

Sample No.1-10 was fractionated according to colors of iodine stain, and according to molecular size (Table I, Figures I and II) when G-100 was used, and the same fractionation pattern was obtained when G-200 (Table II, and Figure III) was used. The following general features regarding the fractionation behaviour of this sample using either G-100 or G-200 may observed.

That the sample behaves according to the molecular seiving properties of sephadex as seen from literature where the largest molecular weight molecules are eluted out of the column first, followed by the next largest molecular weight molecules, followed by the next largest, etc. (Tables I, II, Figures I, II, and III).

That the fractionated fractions gave maximum absorbance over a wide range of wave lengths ranging from 610 mu to 560 mu (See Figure I for example, and also Tables I, II, and Figures II, and III).

That the 5 ml sample containg 20 milligrams of hydrolyzed sample was received in eight fraction (See Figures I, and II) when G-100 was used, and in at least 14 fractions (See Table II) when G-200 was used. The reason of this will be discussed later under separations with G-100 and G-200. However, it should be mentioned that when a 50 milligrams sample was fractionated using G-100 (Table I), the 5 ml sample were fractionated in at least 11 fractions (Table I). This point also will be discussed later.

The fractionation patterns as measured by iodine stain technique were reproducible, both using G-100 as well as G-200. It also has been shown that molecules of the same molecular weights will always appear in the same place, providing that the bed volume, flow rate, hydrostatic pressure, gel type, and temperature are all the same (14,2, 8, 10, 22, 23).

Sample No.2-10 and No.2-20 were fractionated using Sephadex G-200, and results are shown in Table III, and IV. Much of the general features of the fractionation behaviour of this sample is the same as the previous one (Table III, and IV). However, both samples gave their maximum absorbances in the most sensitive region of the iodine stain method (60), and they represent actual dextrins that are suitable for measurements the iodine stain technique. And that is because, no maximum absorbance

was observed to exceed 596 mu (Table III) or 588 mu (Table IV). And thus the insensitive region of the method was avoided, where at higher wave lengths more than 600 mu the method become insensitive.

However, the descrepancy in some of the D.P. values (Table IV) may be attributed to some unexplained reason, because of the unusual behaviour of G-200 (14, 2, 8, 10, 22, 23).

II. Nelson's Method (43)

Nelsons modified method (43) has been used to determine the D.P.'s for those fractions which gave positive iodine stain color (60) for Sample No.1-10 using G-100 (Table I). Stewart and Nordin developed a method (58) for the determination of D.P.'s of both oligosaccharides and dextrins. They determined the total glucose by the anthrone method (59), and the glucose equivalent by Somogyi method (30), and the D.P. is determined from the ratio of total glucose by anthrone to glucose equivalent by Somogyi method (58).

Since Nelson's method is a modification of Somogyi's (28,30), and it is as precise as the titrimetric method (28, 30); and since the phenol-sulfuric acid method (59, 73) determines the total carbohydrates as well as the anthrone method (59), it was then possible to find an extension of this method to dextrins of higher D.P.'s (58) as was recommended by Stewart and Nordin (58).

The observed D.P.'s as measured by this method check well with the corresponding values as determined from the iodine stain technique (60);

and the drawbacks of the method (46) as was reported do not apply here because the sample is a dextrin (58) , and care was taken to exclude air.

Moreover, it should be noted that the method is precise over a wide range, and that it is easy and rapid to operate with highly reproducible results (36, 28, 37, 38, 39, 32, 35). Nelson's method should be used only when the copper-oxidized carbohydrate solution is transparent and relatively coloreless (43, 28).

A 50 milligram sample was fractionated in order to have enough carbohydrate per ml, and 3 ml aliquots were analyzed for Nelson's in order to have readiable optical density units, and to avoid low readings (See Materials and Methods). Total maltose has been calculated using maltose standard of 200 ug/ml. At the same time the phenol-sulfuric acid method (59, 73) was run for analysis of total carbohydrate, and the D.P.'s were calculated for fractions which gave positive iodine stain colors, from the ratio of total carbohydrate by phenol-sulfuric acid method to the total maltose by Nelson's method X 2 (Table I). It was necessary to multiply by 2, because maltose standard curve was used to calculate the total maltose, and maltose has 2 anhydroglucose units.

The D.P. values as determined by Nelson's Method check well with those determined by iodine stain (Table I), and the reason is that the sample used was a dextrin (58), and the analysis has been conducted using Folin tubes with marbles placed in the mouths of the tubes, so that air-oxidation was eliminated (28, 30, 43).

III. Separation with G-100 (14, 2, 72, 8, 10, 23)

G-100 was used to fractionate sample No.1-10, and results are shown in Figures I, II, and Table I. From the results obtained using G-100, it can be said that it is applicable to fractionate commercial dextrins of pyrodextrin type such as British Gum which has an average D.P. of 66 (82). It is also suitable for fractionation of other dextrins which have average D.P.'s within the range 94-41 (Seee Table I, and Figures I, and II).

Under the standard conditions used for the iodine stain technique (60) the fractionation obtained using G-100 is ideal and reproducible. From Figures I, II, and Table I, it is concluded that Sample No.1-10 has been fractionated smoothly according to both colors (Table I, and Figure II), and to molecular size.

G-100 is easily packed (14, 2, 8, 10, 72, 22, 23), and the background can be controlled. Moreover, faster flow rates (72) can be maintained and achieved without running the risk of column stoppage because of compression of the gel (72). Higher hydrostatic pressure could be used also without running the risk of repacking the column.

When the same conditions are maintained, the fractionation patterns obtained will be reproducible and alike. That is to say fractionation is reproducible when the bed volume, flow rate, hydrostatic pressure, the gel type, and temperature are all the same. However, when the sample concentration is changed from 20 milligrams (See Tables III, IV, and Figures I, and II) to 50 milligrams (See Table I) with a change in the flow rate, one property of gel filtration was observed; and that is the sample concentration is a factor controlling fractionation patterns

(14, 2, 8, 10, 72, 22, 23):

1. Because of the change in the sample concentration, the same 5 ml sample has been fractionated into 11 fractions, each of which gave iodine stain colors (Table I). While, the 5 ml sample containing 20 millgrams has been fractionated into only 8 fractions (from No.8 thru No.16 inclusive) (See Figures I, and II). This means that increasing only the sample concentration resulted in obtaining three fractions more. However, this may not mean better fractionation was achieved because of the increase in the sample concentration (See Table I, and Figures I, and II). It tells, however, that sample concentration is a factor controlling the fractionation pattern in gel filtration as seen from the literature (14, 2, 8, 72). The maximum was in the same place in both cases (Table I and Figures I and II).

IV. Separations with G-200 (72, 14, 2, 8, 10, 22)

G-200 has been used to fractionate all samples prepared (See Tables II, III, IV, and Figure III). From the results obtained (Tables II, III, IV, and Figure III) it is obvious that G-200 was as good as G-100 to fractionate starch dextrins of D.P. ranging from 92 to 32 (Tables II, III, and IV, and Figure III). However, its applicability is limited because of:

a. Solubility of Sephadex (14, 2, 8, 10, 22, 23), and because G-200 is less cross-linked (14, 2, 8, 10, 72, 22); it has been observed that the background could not be controlled and elution diagrams using the phenol-sulfuric acid method are not reproducible.

b. The gel is more porous (14, 72, 8, 2, 10, 22), and thus its water regain is twice that of G-100 (10:20) (72, 14, 22). Thus its

ability to swell in aquous solutions is twice as that of G-100 (14,72). This property makes the gel readily compressable (14, 72, 10, 8) when higher hydrostatic pressures are used. Therefore, it has been recommended that a hydrostatic pressure of no more than 10 cm. be used (72). If more hydrostatic pressure is used, we will run the risk of complete column stoppage (14, 72, 22).

c. It should be mentioned that with a low hydrostatic pressure of only 10 cm., only a very slow flow rate could be achieved; and if more pressure has been used in order to mentain a reasonable flow rate, we definitely will run the risk of repacking the column because of complete stoppage. All this amounts to time and effort spent in vain.

d. Moreover, with a very slow flow rate we run another risk. That is to say, amylose samples of higher D.P.'s will tend to retrograde on the column before the fractionation experiment is over. This indirectly will mean that when quantitative analyses are run, recovery of the sample will not be 100%.

Fractionation patterns using G-200 are also reproducible, provided that the bed volume, flow rate, hydrostatic pressure, gel type, and temperature are all the same.

However, it should be noted when the gel type was changed from G-100 (Table I, Figures I, and II) to G-200 (Tables II, III, IV, and Figure III) with a change in the column dimensions from 61 X 1.5 cm. in case of G-100 to 71 X 1.7 cm. in case of G-200, an other property of gel filtration has been observed:

1. The fractionation pattern has been changed, because it has been affected by the change in the gel type, and in the bed volume (14, 10, 2, 72). It should be noted that the 5 ml sample containing 20 milligrams sample has been fractionated differently in both cases. That is to say, when G-200 was used the sample has been collected in at least 12-14 fractions (See Tables II, III, and IV), while when G-100 was used the same sample has been collected in no more than 8 fractions (See Figures I, and II). This could be attributed to:
   a. G-200 swells more in aquous solutions such as water, and its water regain per gram is twice as that of G-100 (72, 14, 10).
   b. The void volume that is held by the same volume of swollen gel is much more in case of G-200 than that of G-100 (72, 14, 10, 2).
   c. The more the degree of cross-linking, the less is the ability of the gel to swell in aquous solutions. This means that low G-Series swell less than higher G-Series (72, 14, 8, 10, 2). This is because most of the pores in the low G-Series are filled with the glyceryl bridges resulting from the cross-linking with epichlorohydrin (14, 10, 2, 22). Since sephadex is available in beads (72), and since the higher G-Series are less cross linked, then any decrease in the particle size (higher figures for sieve fraction mesh) will result in a decrease in the flow rate for the same type of gel (72, 14, 2, 8, 10, 22, 23). That is to say, a G-200 of 100-200 mesh would have faster flow rates than a G-200 of 200-270 mesh (72, 14, 10, 2, 8, 22).

From the above three points, one can conclude that G-200 of the same

mesh size as G-100 would give better fractionation (See Tables II, III, IV, and Figures I, II, and III). However, fractionation using G-200 has the limitation which has been discussed before. Nevertheless, G-200 has been used to fractionate human serum proteins and hemoglobin, but on larger columns (83) where a flow rate of 10-20 ml/hr. could be achieved.

Sephadex could be used for some commercial purposes, such as the production of low lactose milk, from samples of skim milk. However, sephadex of low G-Series such as G-25 could be used, with huge columns (96 liters) which could maintain reasonable flow rates of 4 liters per minute (72).

SUMMARY

By using a column of either G-100 or G-200, it was shown that partialy hydrolyzed samples of amylose were fractionated according to molecular size.

Fractionation patterns are reproducible with G-100 but with G-200 the elution diagrams using the phenol-sulfuric acid method were not reproducible, and the background could not be controlled. In case of G-100 the elution diagrams using the phenol-sulfuric acid method were reproducible, and they took the shape of continuous curves.

Fractionated samples stained with iodine gave colors ranging from blue-green, blue, purple, to red reflecting separation according to molecular size.

The D.P.'s for the collected fractions which gave positive iodine stain colors were determined by Nelson's alkaline copper method and by iodine stain technique. D.P. values obtained check well be both methods.

It was concluded that G-100 is applicable to fractionate commercial dextrins of the pyrodextrin type, such as British Gum. Also, it is suitable to fractionate other dextrins which have average D.P.'s with the range 94 - 41.

REFRENCES

1. P. Flodin and J. Porath, in "Chromatography," E. Heftman, ed., Reinhold Publishing Corp., 99, 101 (1962).

2. J. Porath and P. Flodin, Nature, 183, 1657, (1959).

3. J. Porath and P. Flodin, Nature, 183, 1657- (1959).

4. P. Flodin and K. Aspberg, "Biological Structure and Function," Academic Press Inc., London, England, Vol. 1, 1961, P. 345-

5. K. Granath and P. Flodin, Makromol. Chem., 48, 160, (1961).

6. T. Scott and E. Melvin, Anal. Chem., 25, 1656, (1953).

7. P. Flodin and K. Aspberg, in "Biological Structure and Function," Vol. 1, p.345, Academic Press, London, (1961).

8. P. Flodin, J. Chromatography, 5, 103, (1961).

9. J. Porath and E. B. Linder, Nature, 191, 69, (1961).

10. B. Gelotte, J. Chromatography, 3, 330, (1960).

11. P. Nordin, Arch. Biochem. Biophys., 99, 101 (1962).

12. P. Flodin and K. Aspberg, in "Biological Structure and Function," T. W. Goodwin and O. Lindberg, eds., Academic Press Inc., New York, N. Y., (1961), Vol. 1, P.345-

13. N. R. Ringertz and P. Reichard, Acta Chem. Scand., 14, 303, (1960).

14. K. A. Granath, Makromol. Chem., 28, 1, (1958).

15. G. Oestling, Acta Soc. Med. Upsaliensis, 64, 222, (1960).

16. A. Bill, N. Marsden, and H.R. Ulfendahl, Scand. J. Clin. & Lab. Invest., 12, 392, (1960).

17. R. Montgomery, Abstracts Papers, Am. Chem. Soc., 139, 10D, (1961).

18. K. Granath and P. Flodin, Makromol. Chem., 48, 160, (1961).

19. R. L. Kisliuk, Biochem. Biophys, Acta, 40, 531, (1960).

20. J. K. N. Jones and R. A. Wall, and A. O. Pittet, Can. J. Chem., 38, 2285, (1960).

21. J. K. N. Jones and R. A. Wall, Can. J. Chem., 38, 2290, (1960).

22. P. Flodin and K. Aspberg, "Biological Structure and Function," Academic Press Inc., London, England, Vol. 1, 1961, P.345.

23. N. W. Taylor, H. F. Zobel, N. N. Hellman, and F. R. Senti, J. Phys. Chem., 63, 599, (1959).

24. R. Benthy, Method in Enzymology, 1, 340, (1955).

25. A. O. A. C., Washington, D. C. 8 th Eddition, 1955.

26. R. E. Strange, F. A. Dark, and A. G. Ness, Biochem. J., 59, 172,(1952).

27. H. L. Schlesinger and H. C. Brown, J. Am. Chem. Soc., 62, 3429,(1940).

28. M. Somogyi, J. Biol. Chem. 195, 19, (1952).

29. H. Wanner, Helv. Chim. Acta, 35, 460, (1952).

30. M. Somogyi, J. Biol. Chem., 160, 61, (1945).

31. J. Blom and C. O. Rosted, Acta Chem. Scand., 1, 32, (1947).

32. A. E. Flood, E. C. Rist, and J. K. N. Jones, J. Chem., Soc., 1679, (1948).

33. M. Somogyi, J. Biol. Chem., 117, 771, (1937).

34. H. G. Wager, Analyst, 79, 34, (1954).

35. A. J. Willis and E. W. Yemm, New Phytologist, 54, 289, (1955).

36. E. J. McDonald, Anal. Chem., 29, 32, (1957).

37. S. J. Patterson and R. I Savage, Analyst, 82, 812 (1957); also Die Strake, 11, 70, (1959).

38. K. Heyns, Die Starke, 11, 68, 216 (1959).

39. J. E. Hodge and H. A. Davis, "Selected Methods for Determing Reducing Sugars", U. S. Department of Agriculture, Peoria, Illinois, Bulletin AIC 333, 66 pp. (1952).

40. H. W. Anner, Helv. Chem. Acta, 35, 460, (1952).

41. R. B. Duff and D. J. Eastwood, Nature, 165, 848, (1950).

42. R. S. Schallenberger and R. G. Moores, Anal. Chem., 29, 27, (1957).

43. N. Nelson, J. Biol. Chem., 163, 375, (1944).

44. H. G. Wager, Analyst, 79, 34, (1954); also 81, 483, (1956).

45. L. G. Paleg, Anal. Chem. 31, 1902 (1959).

46. H. F. Launer and Yoshio Tomimatsu, J. Anal. Chem. 33, No.1, January, 1961, P.79.

47. Whistler, R. L., BeMiller, J. N., Advances in Carbohydrates, Chem. 13, 289, (1958).

48. Bottle, R. T. Gilbert, G. A., Greenwood, C. T. Saad, K. N., Chem. and Ind. (London), 541, (1953).

49. Heuser, Emil, "Cellulose Chemistry," pp.472-9, Wiley, New York (1944).

50. P. A. Shaffer and M. Somogyi, J. Biol. Chem., 100, 695, (1933).

51. H. S. Isbell C. F. Snyder, N. B. Holt, and M. R. Dryden, J. Research Natl. Bur. Standards, 50, 81, (1953).

52. I. A. Wolff, C. L. Mehltretter, R. L. Mellies, P.R, Watson, B. T. Hofreifter, P. L. Patrick, and C. E. Rist, Ind. Eng. Chem., 46, 370, 2605, (1954).

53. B. G. Goss, J. Assoc. Offic. Agri. Chemists, 36, 245, (1953).

54. Official Methods of Analysis, Association of Official Agricultral Chemists, Washington, D.C., 8 th Edition, (1952).

55. Isbell, H. S. Pigman, W. W., Frush, H. L., J. Reseach Natl. Bur. Stand., 24, 241, (1940).

56. Dimler, R. J. Wolf, I. A., Soloan, J. W., Rist, C. E., J. Am. Chem. Soc. 77, 6569, (1955).

57. M. Somogyi, J. Biol. Chem. 160, 61(1945); 195, 19, (1952).

58. L. Stewart and P. Nordin, Anal. Chem. 5, 175, (1963).

59. R. J. Dimler et al., Anal. Biochem. 2, 531, (1961).

60. Bailey, J. M. and Whelan, W. J., J. Biol. Chem., 236, 969, (1961).

61. R. M. McCready and W. Z. Hassid, J. Am. Chem. Soc., 65, 1154, (1943).

62. Bates, F. L. D. French, and R. E. Rundle, J. Am. Chem. Soc., 65, (1943).

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

64. Mould, D. L. and Synge, R. L. M., Biochem. J. 58, 571, (1954).

65. Rundle, R. E. et. al., J. Am. Chem. Soc., 66, 2116, (1944).

66. Meyer, K. H. and Bernfeld, Helv. Chim Acta, 24, 389, (1941).

67. Foster, J. F., and Paschall, E. F., J. Amer, Chem. Soc. 75, 1181, (1953).

68. Rundle, R. E. and French, D., J. Am. Chem. Soc., 65, 558, (1943).

69. Rundle, R. E. and French, D., J. Am. Chem. Soc., 65, 2200 (1943).

70. Bates, R. L. et al., J. Am. Chem. Soc., 65, 142, (1943).

71. Rundle, R. E. et al., J. Am. Chem. Soc., 66, 2116-8, (1944).

72. Sephadex in Theory and Practice, Pharmacia, Uppsala, Sweeden, 1967.

73. Dubois, M. Belles, K. A. Hamilton, J. K. Rebers, P.A. and Smith, F., Anal. Chem. 28, 350, (1956).

74. J. E. Hodge and B. T. Hoffeiter, Methods in Carbohydrate Chem., Vol. 1, 380, New York, (1962).

75. R. B. Koch, W. F. Geddes, and T. Smith, Cereal Chem., 28, 424,(1951).

76. M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, Anal. Chem., 28, 350, (1956); Nature, 168, 167, (1951).

77. A. J. G. Barnett and G. A. Tawab, J. Sci. Food Agr., 8, 437, (1957).

78. R. Dreywood, Ind. Eng. Chem., Anal. Ed. 18, 499, (1946).

79. F. L. Bates, et al., J. Am. Chem. Soc. 65, 142, (1943).

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

81. H. F. Launer, and Y. Tomimatsu, Anal. Chem. 31, 1385, (1959).

82. R. L. Whistler, and E. F. Paschall, Starch, Chemistry and Technology, Vol. 2, p. 255 (1967).

83. Killander, J. Biochem. Biophys. Acta, 93, p.1-14, (1964)

84. Y. C. Lee and Rex Montgomery, Methods in Carbohydrate Chemistry, Vol.5, p. 28, (1965).

FRACTIONATION OF STARCH DEXTRINS USING SEPHADEX

by

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AN ABSTRACT OF A MASTER'S THESIS

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Separately, two samples of Commercial amylose (Stein Hall Inc., Superlose) of 20 grams each were dissolved in dimethyl sulfoxide and precipitated with 1-butanol. The first sample (No.1) was air dried before hydrolysis with 200 ml of 0.01 N HCl, while the second sample (No.2) was directly hydrolysed with 250 ml of 0.01 HCl. In both cases hydrolysis was carried under reflux, and samples were withdrawn at different stages of hydrolysis and immersed immediately in an ice bath. The samples were neutralized with 1 N ammonium hydroxide and lyophilized to dryness. The samples were applied to uniformly packed columns of Sephadex G-100 or G-200 (Pharmacia Fine Chemicals, Inc., Box 1010, Rochester, Minnesota; Sephadex in Theory and Practice, Pharmacia, Uppsala, Sweden, 1967), and were collected as 5 ml fractions with a fraction collector. If not ready for analysis, the samples were stored by complexing with few drops of chloroform which was easily expelled by warming.

Total carbohydrate content for the collected fractions was determined by the phenol-sulfuric acid method (Dubois, M. et al., Anal. Chem. 28, 350, 1956), where 1 ml of 5% phenol was added to 1 ml of carbohydrate solution and mixed. 5 ml of concentrated sulfuric acid, reagent grade, were added to each tube and carefully mixed during 10 minutes. The tubes were left to stand either at room temperature or in a water bath at 25° to 35° C for 20 minutes before the absorbance of the yellow-orange color was taken at 490 mu using Bausch & Lomb Aloe Spectrophotometer with refrence to a reagent blank and a standard curve.

Nelson's alkaline copper method (Nelson, N., J. Biological Chemistry, 153, 375, 1944) was used to determine the Degree of Polymerization for the collected fractions which gave positive iodine stain colors. This was carried by adding 1 ml of Nelson's copper reagent to 3 ml of carbohydrate solution with mixing. The Folin tubes with marbles placed in their

mouths, were heated for exactly 20 minutes in a boiling water bath. Then tubes were cooled simultaneously and 1 ml of arsenomolybdate reagent was added to each tube with mixing. After 5 minutes with occasional shaking, each reaction mixture was diluted to 10 ml with distilled water. Absorbances of the developed blue colors were taken at 540 mu using the previous spectrophotometer with refrence to a reagent blank and a standard curve using maltose.

The Degree of Polymerization was also determined by the Iodine Stain Technique (Bailey, J.M. and Whelan, W. J., J. Biol. Chem. 236, 969, 1961). 1 ml of 1 N NaOH was added to 2 ml of carbohydrate solution with mixing, and heated for exactly 3 minutes in a boiling water bath. The 25 ml measuring flasks were simultaneously cooled in a running tap water and 1 ml of 1 N HCl was added to each flask with shaking. This was followed by the addition of 0.05 to 0.07 gram of potassium hydrogen tartrate with mixing. The volume of each flask was put to 22.5 ml with distilled water, before the addition of 0.25 ml of 0.2% iodine in 2% KI. After shaking, each flask was put to final volume of 25 ml with distilled water and mixed. After 20 minutes, the wavelengths of maximum absorbance for the developed colors were measured using Backman DB or DU Recording Spectrophotometer in the visible range from 760 mu to 320 mu, with refrence to a reagent blank and the Iodine Stain Empirical Method.

The text contained an introduction, pertinent literature cited, the results obtained, discusions, and refrences. It was concluded that Sephadex G-100 is suitable for the fractionation of Commercial dextrins of the pyrodextrin type, such as the British Gum.