GEL DIFFUSION CHROMATOGRAPHY OF DEXTRAN

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INTRODUCTION

Clinical dextran preparations, in order to be physiologically effective, must fall within a rather narrow molecular weight range in the neighbourhood of 100,000. Physico-chemical evaluation of such preparations is usually performed by time-consuming fractional precipitation with alcohol, followed by measurement of the molecular weight of the fractions by light scattering or viscosimetry. A chromatographic method has been developed whereby an estimate can be made of the relative proportions of the dextrans of various molecular weights in a heterogeneous mixture using less than 100 mg of sample.

The method described below is similar in principle to chromatography on starch columns as described by LATHE AND RUTHVEN[1] and to that of gel filtration described by PORATH AND FLODIN[2]. The latter method was recently extended[3] to the separation of dextrans of molecular weights below 10,500. To date no satisfactory separation of dextrans in the molecular weight range of 30,000 to 500,000 has been reported with either Sephadex* or starch columns although in the latter case separation of amylopectin (mol. wt.: 10^6) from hemoglobin (mol. wt. 67,000) was achieved[1]. Agar was investigated as a medium for gel diffusion chromatography because it is readily available and forms gels whose physical properties are amenable to modification by changing concentration or pH, or by employing additives. The investigation was chiefly exploratory, its aim being to discover the optimum conditions for an evaluation of dextran preparations.

MATERIALS AND METHODS

(1) *Preparation of agar*

Bacto-agar** (1 lb.) was agitated with approximately 25 l of tap water overnight and then allowed to settle. The yellow turbid supernatant was decanted and the residue repeatedly washed with tap water by decantation until the supernatant was clear and colourless. The agar was then recovered by filtration on a coarse sintered glass funnel, washed with distilled water and ethanol and finally air dried. The dried, lumpy material was ground to a coarse powder in a Wiley mill and stored in closed bottles.

(2) *Preparation of agarose*

Agarose acetate was prepared from agar using the method of ARAKI[4–7]. Thirty grams

* Trade name for cross-linked dextran gels manufactured by Pharmacia, Uppsala, Sweden.
** Difco Laboratories, Detroit 1, Mich., U.S.A.

of Bacto-agar (prepared as in (1) above) ground to a fine powder in a ball mill (for 19.5 h), were mixed with 200 ml of an 80 % solution of pyridine in water, and the mixture was kept at 60° for approximately 5 h to allow the agar to swell. To the cooled mixture were added pyridine (100 ml) and acetic anhydride (500 ml) and the mixture was agitated at approximately 70° overnight. The turbid, syrupy mixture was then poured slowly with rapid stirring into 10 vol. of ice and water. A stringy precipitate formed at first but continued stirring reduced it to granular particles. When the ice had melted the precipitate was allowed to settle, most of the supernatant was drawn off and discarded, and the precipitate collected and thoroughly washed with water on a Büchner funnel. The product was dried first in an air oven at 60° and then in a vacuum oven at 60° overnight, yielding 33 g of fibrous, white agar acetate. The latter was reduced to a powder and stirred with 1 l of chloroform*. A portion of the powder appeared to dissolve rapidly whereas the remainder was undissolved after prolonged stirring. Filtration presented great difficulties due to the gummy nature of the insoluble portion (agaropectin) and caused much loss of material. It was found most convenient to use 50 cm fluted filter papers (Eaton-Dikeman No. 541) and to change these when the filtration became slow. To the filtrate was added with stirring an equal volume of petroleum ether (Skellysolve B). The precipitate which formed was collected on a fine sintered glass funnel, washed with petroleum ether and dried in the air. Yield 8.6 g of agarose acetate. Agarose was prepared by catalytic deacetylation of agarose acetate with sodium[8–10] as follows: To agarose acetate (8.6 g) suspended in anhydrous methanol (350 ml) was added a small piece of sodium (approx. 100 mg). The mixture was stirred overnight at approx. 40° and filtered through a sintered glass funnel. The dark yellow filtrate was discarded and the residue washed with methanol and dried. Yield of nearly white powder: 5.8 g (agarose). A hot 1 % aqueous solution of this material set to a stiff gel on cooling.

(3) *Preparation of agar and agarose gel columns*

Gels were prepared by suspending powdered agar (6–9 %) or powdered agarose (4 %) either with or without powdered cellulose (passing 200 mesh; approx. 3 %) in water or buffer, autoclaving the mixture at 15 lbs. for 15 min and cooling it. When cellulose was used the mixture was agitated just prior to gelation in order to distribute the fibers as uniformly as possible. When the gel had thoroughly hardened, it was reduced to fine particles in a Waring or Braun homogenizer in the presence of water. The particles were graded by wet sieving using a jet of distilled water, material passing 20 mesh but not 40 mesh being retained. The sieved agar was suspended in distilled water or buffer and poured by increments as a slurry into a water jacketed column. As the particles settled packing was facilitated by vibration of the column with an electric motor and by slow escape of liquid from the stopcock at the bottom. When packing was completed the column was irrigated with eluant until only a trace of colour was produced on testing 2 ml of the effluent by the phenol–sulphuric acid procedure (see (8) below). In some cases where other than room temperature was used, sufficient time was allowed for the interior temperature of the column to reach that of the jacket before chromatography was begun.

* Acetone may be used instead of chloroform; in this case a coarse sintered glass funnel may be used for filtration; yield, 17.7 g of agarose acetate from 31.6 g of acetylated agar.

(4) *Preparation of Cyanogum (polyacrylamide) gel columns*

A 10 % Cyanogum gel was prepared as follows. Cyanogum 41* (10.0 g) was dissolved in 89 ml of distilled water and the solution was filtered. Dimethylaminopropionitrile (DMAPN catalyst, 0.4 g) and potassium ferricyanide (0.015 g) were dissolved in the filtrate. To initiate polymerization, 0.50 g of ammonium persulphate was dissolved in the solution and the latter was allowed to stand until gelation was complete (30 min). Other gels containing 5 %, 7.5 %, and 15 % Cyanogum 41 by weight were prepared by using more or less Cyanogum 41 as above, except that the weights of DMAPN, potassium ferricyanide and ammonium persulphate relative to the weight of Cyanogum 41 were held constant. In some preparations potassium rather than ammonium persulphate was used, in which case the appropriate stoichiometric correction was made and the potassium salt was added in solution rather than as a solid. Chromatographic columns of Cyanogum were prepared in the same manner as agar and agarose columns.

(5) *Preparation of Sephadex column*

Sephadex G-50** (20 g) was suspended in 3 l of distilled water and allowed to settle. Fine particles were removed by decantation and the procedure repeated twice. The Sephadex slurry was packed into a column and washed in the same manner as agar.

TABLE I

Dextran Type	*Condition*	*Source*	*Mol. wt. (in thousands)*		
			Average	*Highest 5–10 % by wt.*	*Lowest 5–10 % by wt.*
G-2	Powder	Company A	543		
BD-1	Powder	This laboratory*	357		
G-3	Powder	Company A	277		
BD-5	Powder	This laboratory	62.5		
G-9	Powder	Company A	30.2		
DB	Powder	Company A	approx. 5		
H	Powder	Company A	partially hydrolysed, unfractionated		
B-959	6 % Clinical soln.	Company B	97	154	30
B-1958	6 % Clinical soln.	Company B	128	417	25
G-60-110	6 % Clinical soln.	Company A	111	186	26

* Fractionation and molecular weight determination carried out by Dr. W. D. GRAHAM, formerly of this laboratory.

(6) *Dextran preparations*

Table I lists the dextran preparations used in the present study. All molecular weight determinations were made by light scattering photometry and fractions were prepared by precipitation with alcohol[11].

* Obtained from: Cyanamid of Canada Limited, 635 Dorchester Boulevard West, Montreal 2, Que.

** Obtained from: Pharmacia Laboratories Inc., 501 Fifth Avenue, New York 17, N.Y.

(7) *Chromatography*

Dry samples of dextran or glucose (10 to 50 mg of each) were dissolved in 1.0 to 1.5 ml of eluant and applied to the column. Samples of clinical dextran (0.3 or 0.7 ml of 6 % solutions in physiological saline) were placed directly on the column. Elution was carried out using distilled water or buffer (as indicated later) to which had been added a trace of phenol to prevent growth of microorganisms. The fractions were collected automatically*.

(8) *Carbohydrate determination*

The method of DUBOIS *et al.*[12], employing phenol and sulphuric acid was used to determine glucose and dextran. In most cases an Evelyn colorimeter with a No. 490 filter was used, but occasionally when absorbance values were very high a No. 540 filter was used. Appropriate standard curves were constructed in each case.

RESULTS AND DISCUSSION

(1) *Scope of the present study*

The following data are selected from some 58 chromatographic experiments. Information on the influence of the following factors upon the degree of separation of dextrans of different molecular weights was obtained: (a) type of gel, (b) molecular weights of dextrans undergoing separation, (c) nature of eluting agent, (d) rate of elution, (e) temperature, (f) amount of dextran in relation to cross section of column (loading), (g) gel particle size, and (h) column length.

(2) *Agar columns*

It was possible to use columns containing as little as 6.7 % agar for several weeks provided 3.3 % cellulose was incorporated into the gel to give mechanical strength. 3 % agar gels were tried but discarded when it was found they produced columns which packed down and blocked after a few days. The highest practical concentration of agar is 9 %; a gel of this concentration is extremely stiff and difficult to reduce in the homogenizer, but produces a column with rapid flow rate and not susceptible to blocking on continued use. Increasing the gel concentration from 3 to 9 % improved the separation between BD-5 and glucose but reduced that between dextran BD-1 and BD-5 when the three components were chromatographed together at room temperature using water as eluant. Resolution of the components at 5° was somewhat superior to that at room temperature on 9 % agar with water as eluant. Resolution was poorer with either aqueous acetic acid (pH 3.0) or borate buffer (0.05 *M*, pH 8.0) than with water alone; an improvement was found with the use of Tris buffer, (pH 8.0, see Figs. 2 and 3). Resolution was much improved by changing the column dimensions from 1.67 $cm^2 \times 58.5$ cm (used in the preliminary experiments reported above) to 1.18 $cm^2 \times 145$ cm. Fig. 1 shows data obtained with the longer column at 5° using 9 % agar gel (prepared in water) and eluting with water. It can be seen that a mixture of dextrans BD-1, BD-5 and glucose resolved as three over-lapping components, the glucose appearing last (Fig. 1(a)). Re-chromatography of combined fractions 19, 20, 21, 30, 31, and 32 yielded an elution pattern with two accentuated peaks but with no

* Automatic collector made by Gilson Medical Electronics, Madison, Wisc., U.S.A.

peak corresponding to glucose (Fig. 1(b)). It is evident that some fractionation had taken place. Using the same conditions a sample of partially hydrolyzed unfractionated dextran (preparation H) gave curve (c) (Fig. 1) resembling the left-hand portion of curve (b).

Since the swelling of agar in water is a function of pH with an optimum at approximately pH 8.0[13], it was thought possible to increase the "pore size" of the gel network by preparing the gel with 0.05 *M* tris-(hydroxymethyl)-aminomethanol–HCl buffer of pH 8.0 and eluting with the same buffer. Chromatography of a mixture of

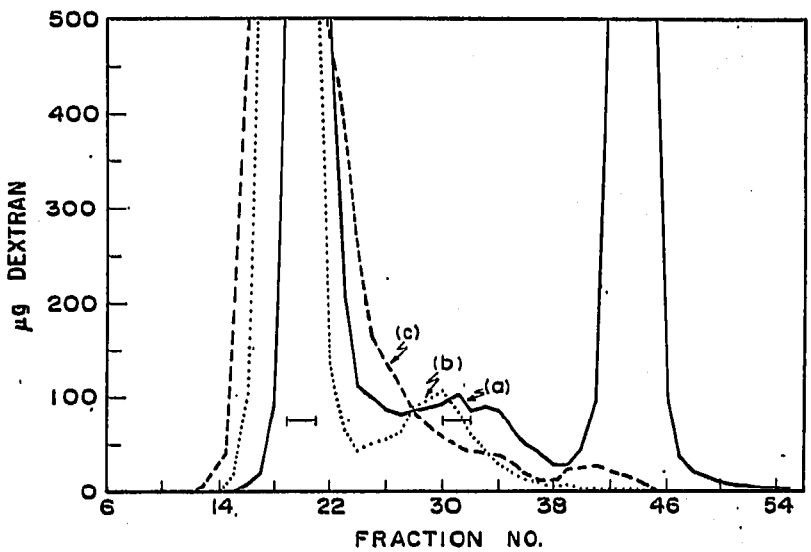

Fig. 1. Column: ht. 145 cm, cross-section 1.18 cm². Gel: 9 % agar, eluted with water. Elution rate: approx. average 7.7 ml/cm²/h. Temp: 4°. (a) Charge: 10 mg of each of BD1, BD5 and glucose. Aliquot for assay: 163 µl; (b) Charge: Concentrated solution of fractions 19–21, 30–32 from previous run (see (a)). Aliquot for assay: 163 µl. (c) Charge: 50 mg H. Aliquot for assay: 337 µl.

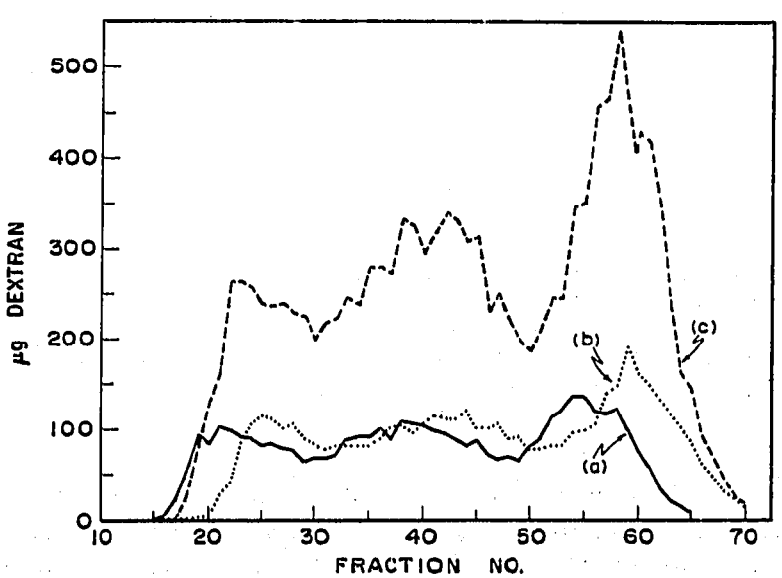

Fig. 2. Column: ht. 145 cm, cross-section 1.18 cm². Gel: 6.7 % agar, 3.3 % cellulose, made up in 0.05 *M* Tris buffer, pH 8.0, and eluted with buffer. Elution rate: approx. average 15.6 ml/cm²/h. Temp.: 4°, Aliquot for assay: 333 µl. (a) and (b) Charge: 10 mg of each of G3, G9 and glucose. (c) Charge: 50 mg of each of G3, G9 and glucose.

dextrans G-3, G-9 and glucose using this gel and buffer system (4°; column 1.18 cm^2 × 145 cm) gave the results shown in Fig. 2. It is apparent that there is partial resolution into three main components. Curves (a) and (b) were obtained under identical conditions with 10 mg of each constituent and do not differ significantly in shape. The displacement of curve (b) relative to (a) can be attributed to an error in numbering

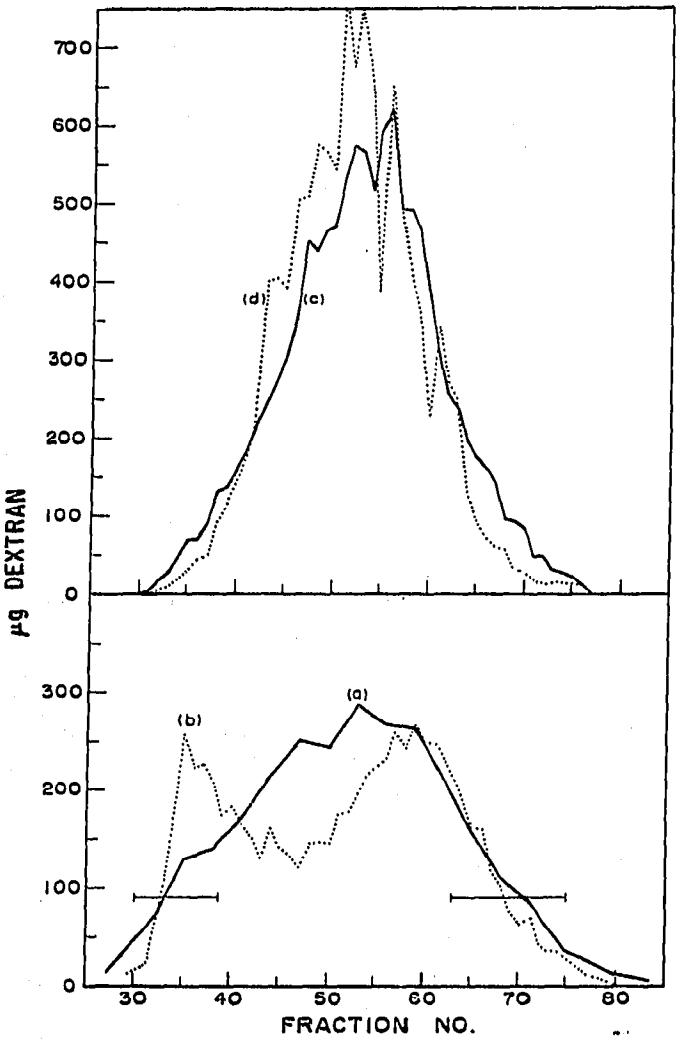

Fig. 3. Column: ht. 145 cm, cross-section 1.18 cm^2. Gel: 6.7% agar, 3.3% cellulose, made up in 0.2 *M* Tris buffer, pH 8.0, and eluted with buffer. Elution rate: approx. average 9.5 ml/cm^2/h. Temp.: Room temp. (a) Charge: 150 mg H. Aliquot for assay: 70 µl. (b) Charge: Dextran precipitated from fractions 30–39, 63–75 from previous run (see (a)). Aliquot for assay: 331 µl. (c) Charge: 1 ml B-1958 ≡ 60 mg dextran. Aliquot for assay: 238 µl. (d) Charge: 1 ml B-959 ≡ 60 mg dextran. Aliquot for assay: 238 µl.

the tubes. Curve (c) was obtained using five times as much of each constituent and shows that the capacity of the column was not exceeded even with 150 mg of carbohydrate. The result of chromatographing the hydrolysed unfractionated dextran (preparation H) on a column of alkaline agar is shown in Fig. 3. The conditions used were the same as those in obtaining the data of Fig. 2 except that the gel was prepared and later eluted with 0.2 *M* rather than 0.05 *M* buffer. Fig. 3(a) shows the initial

fractionation, whereas (b) represents a re-chromatography of combined fractions 30–39 plus 63–75. It is evident that separation into two main components has taken place.

From a comparison of Fig. 3(a) with Fig. 1(c) it is clear that by decreasing the agar concentration from 9 to 6 % and using an alkaline buffer the retention volume for dextran was greatly increased and the distribution pattern altered markedly. Clinical dextran preparations (B-1958 and B-959) produced elution patterns (Fig. 3, curves (c) and (d) respectively) with maxima which fall between the two maxima of curve (b) as would be expected since curve (b) was obtained employing only the fractions containing material of high molecular weight and low molecular weight, while clinical dextran discards, by fractionation, the very high and very low molecular weights, concentrating near the average value of 100,000. Dextran B-959 had previously been found by alcohol fractionation and light scattering to be a more homogeneous preparation than B-1958 (see Table I). This finding is substantiated by the shapes and positions of the elution patterns. Curve (c) is broader than (d) indicating the presence of a higher proportion in the former of components differing markedly in molecular weight from the average.

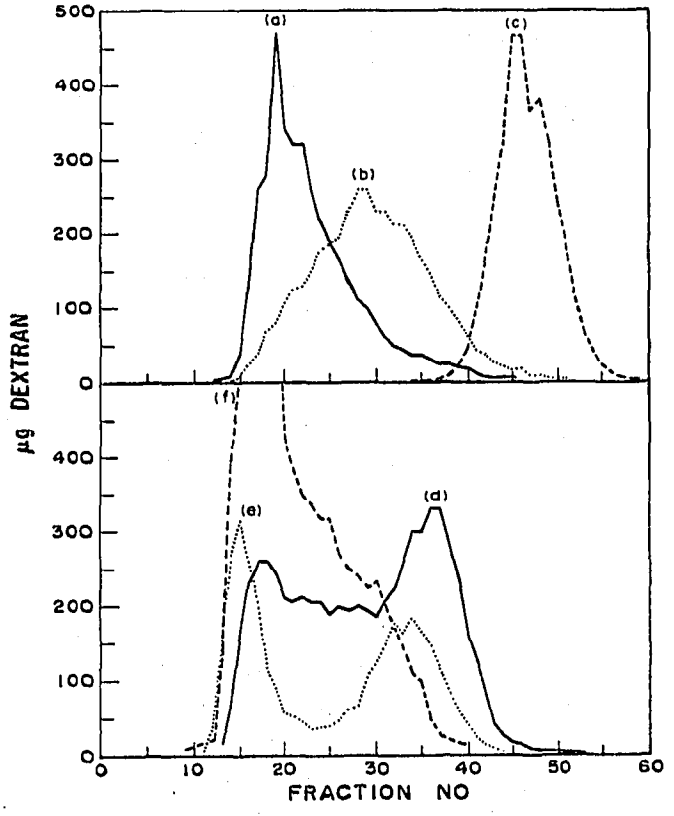

Fig. 4. Column: ht. 62 cm, cross-section 0.79 cm^2. Gel: 4 % agarose, eluted with water. Elution rate: approx. average 17.0 $ml/cm^2/h$. Temp: Room temp. (a) Charge: 10 mg BD1. Aliquot for assay: 666 μl. (b) Charge: 10 mg BD5. Aliquot for assay: 666 μl. (c) Charge: 10 mg glucose. Aliquot for assay: 666 μl. (d) Charge: 10 mg of each of BD1, BD5 and glucose. Aliquot for assay: 333 μl. (e) Charge: 10 mg of each of G2 and DB. Aliquot for assay: 333 μl. (f) Charge: 333 μl G-60-110 ≡ 20 mg dextran. Aliquot for assay: 666 μl.

(3) *Agarose columns*

According to ARAKI[7] agar is composed of at least two components, agarose (linear component capable of forming a gel at 0.35 % concentration) and agaropectin (branched component requiring a minimum concentration of 1.10 % for gelation). Again with the object of increasing the effective "pore size" of the gel network we turned to agarose, on the assumption that the removal of agaropectin from commercial agar might facilitate more rapid diffusion of relatively large dextran molecules into the interior of gel particles. It was found possible to prepare satisfactory columns with 4 % agarose alone, whereas with 6 % agar it was necessary to use cellulose to impart mechanical strength*. Some results obtained with agarose are shown in Fig. 4. Dextrans BD-1, BD-5 and glucose were chromatographed separately on a small column (0.785 $cm^2 \times 62$ cm) at room temperature using water as eluant. The three carbohydrates emerged at distinctly different elution volumes (curves (a), (b) and (c)). As might be expected from their individual elution patterns, BD-1 could be only partially separated from BD-5 when the two preparations were chromatographed together, (curve (d)). To determine whether the poor separation was due to too rapid elution, the experiment was repeated at almost double the rate; essentially the same elution pattern was obtained. Dextrans G-2 and DB, being of widely different molecular weights were almost completely separated when chromatographed together (curve (e)). A sample of clinical dextran (G-60-110) showed an elution pattern (curve (f)) falling in the region expected for such a preparation. It apparently contained a relatively large proportion of material of high molecular weight.

(4) *Sephadex column*

With Sephadex G-50 a complete, sharp separation of dextrans (BD-1 and BD-5) from glucose took place but no resolution of the two dextrans was observed. A column prepared from sieved agar which had been allowed to swell in water (rather than being allowed to gel from solution) produced essentially the same result as with Sephadex G-50. Desalting, "dialysis" or buffer interchange, operations which are readily carried out with Sephadex, perhaps could be carried out with ordinary agar, provided extremes of pH are avoided.

(5) *Chromatography on Cyanogum columns*

Cyanogum gels containing 5 % Cyanogum 41 could not be used since the gel particles were forced through the glass wool plug at the bottom of the column. At lowest practical gel concentration, (7.5 %) complete separation of dextran from glucose was achieved but no significant resolution of the two dextran components, G-3 and G-9, took place (Fig. 5(a)). Essentially the same results were obtained with 15 % Cyanogum (Fig. 5(b)), except that the emergence of the dextrans was markedly retarded, suggesting that the "pore size" of this gel was greater than that of the 7.5 % gel. Very little change in the pattern occurred when the temperature of the column was increased to 60°.

* Since agarose represents approximately 70 % of agar[7], a 4 % agarose gel corresponds in agarose content to approx. 6 % unfractionated agar gel.

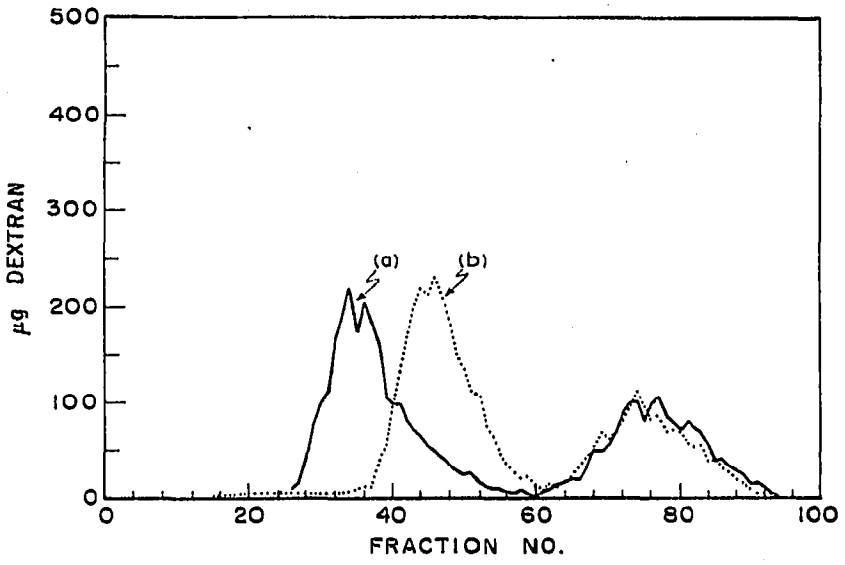

Fig. 5. Column: ht. 145 cm, cross-section 1.18 cm^2. Elution rate (water): approx. average 14.9 $ml/cm^2/h$. Temp: Room temp. Charge: 10 mg of each of G3, G9 and glucose. Aliquot for assay: 331 μl. (a) Gel: 7.5 % Cyanogum. (b) 15 % Cyanogum.

GENERAL CONCLUSIONS

The foregoing data indicate that with columns of agar or agarose gel it is possible to achieve a significant separation of dextrans over the molecular weight range encountered in clinical dextrans. A rough estimate of the molecular weight distribution in a dextran preparation can now be made semi-automatically in one day using less than 100 mg of polysaccharide. The most satisfactory separations were achieved with agar at pH 8.0. The efficiency of separation was not greatly influenced by eluant flow rate, temperature or amount of polysaccharide within wide limits. Tendency of the gel particles to pack down in the column, reducing the rate of eluant flow limited the choice of gel particle size, hence the influence of this factor was not studied.

The most serious limitations of the present chromatographic system are: (a) the low degree of resolution for dextrans of molecular weight above 200,000 with the consequent compression of the early parts of some of the elution patterns, and (b) the semi-quantitative nature of the results obtained. Evaluation of the various gel columns would have been made more directly and more quantitatively if our series of dextran fractions had been monodisperse or at least of very narrow range in molecular weight. With further development of this method it may become possible to obtain a series of highly homogeneous dextran fractions which, after determination of their molecular weights by some independent method, could be used as reference standards in the chromatographic determination of the molecular weight distribution in unknown dextran preparations. The saving of time and materials and the relative simplicity of this method should recommend it to those currently using the alcohol fractionation procedure. Much time is required by a skilled operator to obtain a complete distribution curve by the latter procedure.

Our method may prove of value to those investigating the rate of disappearance from the bloodstream or appearance in the urine of various injected dextran preparations. Frequently, in such studies, only very small quantities of dextran can be recover-

ed from experimental animals and a semi-micro procedure, such as we have described, for their evaluation might be useful.

It should be emphasized that these investigations were of an exploratory nature, designed to discover the limitations of gel diffusion chromatography as a tool for the resolution and analysis of mixtures of high polymers. Further work, particularly with other gel–buffer combinations and with colloidal systems other than dextran, might prove highly rewarding.

While this paper was in preparation a short communication appeared on similar work by POLSON[14]. It is interesting to note that the results are comparable, although POLSON was working with proteins of varying molecular weights while the present work was with the polysaccharide, dextran. In both cases a 7 % agar gel with slightly alkaline buffer elution was found to give the best results.

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SUMMARY

Glucose and dextrans of molecular weight 5,000 to 300,000 can be partially separated chromatographically on columns of agar (6–9 %) with buffer (pH 8.0) or water as eluant. Agarose gels (4 %) can also be used with water as eluant. The method can be used to estimate approximately the molecular weight distribution in small samples of clinical dextrans.

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