Journal of Chromatography, 103 (1975) 49–62
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CHROM. 7891

AGAR DERIVATIVES FOR CHROMATOGRAPHY, ELECTROPHORESIS AND GEL-BOUND ENZYMES

III. RIGID AGAROSE GELS CROSS-LINKED WITH DIVINYL SULPHONE (DVS)

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(Received August 20th, 1974)

SUMMARY

Agarose cross-linked with divinyl sulphone (DVS) is a new matrix for chromatography and immobilized enzymes that has distinct advantages over common agarose gels. It has outstanding mechanical stability as compared with these gels, and the rigid gel beads form beds permitting very high flow-rates. In addition, DVS-agarose is superior to agarose gels with respect to chemical stability in acid and neutral media. In alkaline solutions above pH 8, there is a slow elimination of the sulphone-containing bridges, but without noticeable concomitant dissolution of the gels below pH 12 for moderately or highly cross-linked gels. The DVS-agarose is sufficiently thermostable to be heated in an autoclave.

INTRODUCTION

In previous papers in this series[1,2], we have pointed out the advantages and shortcomings of agar and agarose derivatives as molecular sieves, adsorbents and protein carriers for electrophoresis as well as for electrofocusing. We have also shown how to solve some of the problems. Thus, by cross-linking the gel matrix in reducing medium containing bisoxiranes or halomethyloxiranes (epihalohydrins), products can be obtained that are insoluble in boiling water and hot alkali. An efficient reduction of the number of sulphate-ester groups can be achieved by alkaline hydrolysis at elevated temperature in reducing media. A further reduction in charge can be made by treating the gels with lithium aluminium hydride in dioxane.

Agar, and especially agarose, forms gels of comparatively high rigidity even at low concentrations; this is a prerequisite for efficient use in chromatographic work. Although superior in most other aspects, the agarose gels can not compete in rigidity with porous glass and other ceramics. The elasticity of the beads of low agarose concentration sets the upper limit for particle permeation, and the compaction of the gel bed is likely to be a severe obstacle to large-scale chromatography and in enzyme-bed reactors. To circumvent the bed-elasticity problem we have now developed a new type of agarose-based matrix.

EXPERIMENTAL

Materials

Sepharose gels and Blue Dextran 2000 were obtained from Pharmacia Fine Chemicals AB (Uppsala, Sweden); powdered agarose was a gift from the same company. "Standard chemicals" were all analytical-grade reagents. Orcinol was obtained from E. Merck (Darmstadt, G.F.R.), divinyl sulphone (DVS) purum from Fluka AG (Buchs, Switzerland), human serum albumin (99% pure) from Kabi (Stockholm, Sweden), bovine thyroglobulin Type I from Sigma (St. Louis, Mo., U.S.A.) and tobacco mosaic virus (TMV), *Escherichia coli* cells and ribosomes (70S) were gifts from Dr. Lars Rudin, Dr. Sture Brishammar and Dr. Leif Isaksson, respectively, whose kindness is gratefully acknowledged.

Chemical analysis

Carbohydrates. These were determined by the orcinol–sulphuric acid method[3].

Sulphur and nitrogen. These analyses were performed at the Institute of Analytical Chemistry.

Residual negatively charged groups. Such groups in the gels were determined in the following way:

Approximately 5 g of wet gel were washed on a glass filter, first with water and then with 25 ml of 1 *M* hydrochloric acid. The gel was then washed with distilled water until it was neutral and the washings were chloride-free (tested with silver nitrate). The gel was weighed in the titration vessel, together with 5 ml of 2 *M* potassium chloride, and titration was performed with 0.01 *M* sodium hydroxide with use of a pH-meter (pH Meter 26, Radiometer, Copenhagen, Denmark). The titration curve was drawn, and the estimated inflection point was taken as the equivalence point.

Vinyl groups. The method used for determination is based on the following reaction[4]:

$$R\text{-}SO_2\text{-}CH = CH_2 + S_2O_3^{2-} + H_2O \rightarrow R\text{-}SO_2\text{-}CH_2\text{-}CH_2\text{-}S_2O_3^{2-} + OH^- \quad (1)$$

Two millilitres of 3 *M* sodium thiosulphate were added to 2 ml of cross-linked gel in water. The hydroxyl ions formed were titrated with 0.1 *M* hydrochloric acid using pH-stat equipment (Type TTT 1c, Radiometer, Copenhagen); the pH of 1.5 *M* sodium thiosulphate (5.5) was considered to be the end point of titration. The reaction rate was quite low (10–15 h were needed), and often the reaction mixture was left overnight and titrated the next day.

Preparation of agarose beads

Hot agarose solutions of the desired concentrations were emulsified in a nonpolar solvent with the aid of a suitable emulsifying agent as described by Hjertén[5]. Before use, the beads were sieved to give a size fraction in the bead-diameter range 45 to 250 μm.

In the optimization experiments, all the important variables for cross-linking were kept constant except for the one under test. The cross-linking method used, unless otherwise stated, was as follows:

The wet agarose gel (*a* g; for exploratory studies, *a* was usually 10 or 20, other-

wise it has been 200 or more) was suspended in *a* ml of 0.5 *M* sodium carbonate buffer of pH 11.0. Then DVS was added in an amount expressed as % (v/w) of the wet gel, and the reaction was allowed to proceed for 2 h at room temperature (23°) and 2 h at 45°. The gel was then washed with distilled water until it had neutral pH.

Deactivation of cross-linked gels

The DVS-treated gels contain unreacted vinyl groups, which react with substances containing amino-, hydroxy- or mercapto-groups; the gel should be deactivated with such a substance. 2-Mercaptoethanol in neutral or slightly alkaline medium removed all vinyl groups that could be detected by titration with sodium thiosulphate solution. To *a* g of gel (wet weight) (*a* being 10–200) suspended in a total volume of 2 *a* ml, 0.01 *a* ml of 2-mercaptoethanol was added, and the reaction was allowed to proceed overnight; the excess of 2-mercaptoethanol was removed by washing with distilled water or an appropriate buffer solution.

Amines, such as glycine, can also be used to remove the vinyl groups. The following procedure is recommended:

Glycine solution (10%, w/v, in 1 *M* sodium carbonate) is adjusted to pH 10.0 with concentrated sodium hydroxide solution. The gel is transferred to distilled water, and, for a given amount of settled gel, the same amount of the glycine solution is added and allowed to react overnight at room temperature. The gel is then washed for several hours with buffer solutions of pH 10 and 4 containing 1 *M* sodium chloride and finally with distilled water or the buffer solution for storage or later use.

Flow-rate measurements

The gel (200 ml) was packed in 0.9% sodium chloride solution under low pressure in a 2.5-cm × 50-cm tube to a bed-height of 40 cm. Liquid was delivered from a Mariotte flask kept at a pre-determined height above the column outlet. By this simple arrangement, the steady flow-rate was measured for the given hydrostatic pressure. The time for attaining flow-rate equilibrium was usually 0.5–1 h, except for the 0.5% agarose gels, which required several hours.

Dibasic acid chlorides as cross-linking agents

Sepharose 2B (200 g wet weight) was washed sequentially with 400 ml of ethanol, 400 ml of dioxane and 400 ml of 1,2-dichloroethane containing 10% (v/v) of pyridine. The gels were then suspended in 200 ml of the pyridine–dichloroethane solvent, and, in four identical experiments, pyridine–dichloroethane solutions of 10 g each of succinic, glutaric, adipic, and terephthalic acid chlorides were added dropwise in the cold (5°); the reaction was allowed to proceed overnight in the cold. The next day, the gels were sequentially washed with dichloroethane, ethanol and water, then passed through sieves giving a fraction with particle sizes between 45 and 250 μm. The cross-linked gel beads did not change their appearance when placed in boiling water.

Chromatography

The molecular-sieving properties of the gels were studied by chromatographic experiments on beds packed in Pharmacia Fine Chemicals AB Column Tubes Type K16/100 or K15/90. Elution was performed at constant speed by a peristaltic pump (our own construction). The eluate was passed through the cell of Uvicord Type

4701 A (LKB Products AB, Stockholm, Sweden). The void volume of the bed was determined by using Blue Dextran 2000.

RESULTS

Optimization of cross-linking reaction

Hydroxyl ion concentration. Portions of 20 g of Sepharose 4B were suspended in 20 ml of buffer solution and allowed to react with 0.1 ml (= 0.5 %) of DVS for 1 h at 0°, 1 h at 25° and finally 1 h at 40°; sodium phosphate solution (0.2 *M*) was used at pH 8.0 and sodium bicarbonate–sodium carbonate buffers at higher alkalinities. Complete cross-linking of the carbohydrate chains should convert the gel into a totally insoluble matrix; the extent of cross-linking can therefore be measured by extraction with hot water. After reaction with DVS, the gels were washed on glass filter funnels with 200 ml of boiling water under identical conditions. Dissolved carbohydrate was determined on aliquots of the percolates, and the total amount of matrix substance extracted was calculated and plotted as a function of the pH at which the DVS reaction was carried out (see Fig. 1). As shown in Fig. 1, progressive insolubilization takes place above pH 9, and at pH 11 a gel of extremely low solubility is obtained. The cross-linked gel is sufficiently temperature-resistant to permit heating in an autoclave for reasonable periods.

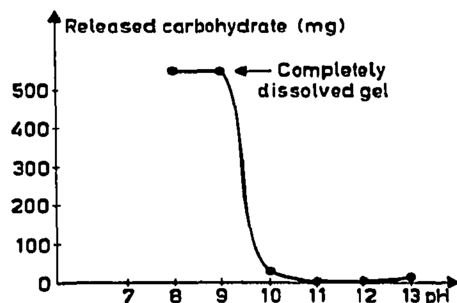


Fig. 1. The cross-linking reaction. Dependence on pH. Sepharose 4B was reacted with 0.5% of DVS (%, v/w, wet gel) at different pH values. The efficiency of the cross-linking reaction was estimated by determination of released carbohydrate by washing the cross-linked gels with boiling water. Details are given in text.

Time and temperature. Portions of 10 g of wet-settled Sepharose 2B were transferred with 10 ml of 1 *M* sodium carbonate buffer of pH 11 to test tubes specially designed to fit the Temperature Gradient Incubator (Scientific Industries Inc., Mineola, N.Y., U.S.A.). After attainment of temperature equilibrium, 25 μl (= 0.25 %) of DVS was added to each tube, and reaction was allowed to proceed for a pre-determined time, with continuous shaking. The gels were then immediately washed (by suction on glass filters) with four 20-ml portions of boiling water; the washings were collected, diluted to 100 ml and analyzed for carbohydrate.

It was found that DVS reacts much faster with agarose at 25° and 40° than at 0°; a reaction time of 1 h is sufficient for max. insolubilization (see Fig. 2).

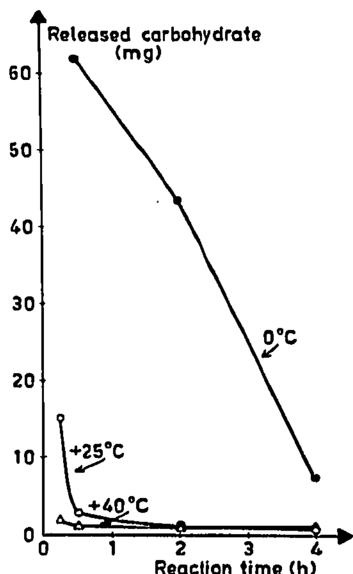


Fig. 2. The cross-linking reaction. Dependence on time and temperature. Sepharose 2B was cross-linked with 0.25 % of DVS at pH 11.0 and at various temperatures for various times. After the reaction, the gel samples were immediately washed with 10 volumes of boiling water, and the amount of carbohydrate extracted was determined. The total amount of carbohydrate in the gel samples was approx. 140 mg.

Stability at low degree of cross-linking

Sepharose 2B and 4B were allowed to react with various amounts of DVS in the concentration range 0–0.4 % (v/w), the products were washed on glass filters with 200 ml of boiling water, and the washings were analyzed for carbohydrate. As can be seen from Fig. 3, gel treated with DVS at a concentration as low as 0.2 % is not very soluble in hot water.

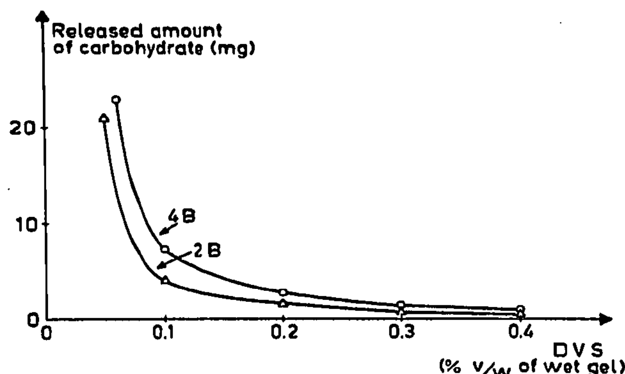


Fig. 3. Cross-linking with low concentrations of DVS. Sepharose 4B and 2B were cross-linked with low concentrations of DVS. After the reaction, the gels were washed with 10 volumes of boiling water, and the water was analyzed for carbohydrate. The amounts (dry weight) of Sepharose 4B and 2B were about 550 mg and 220 mg, respectively.

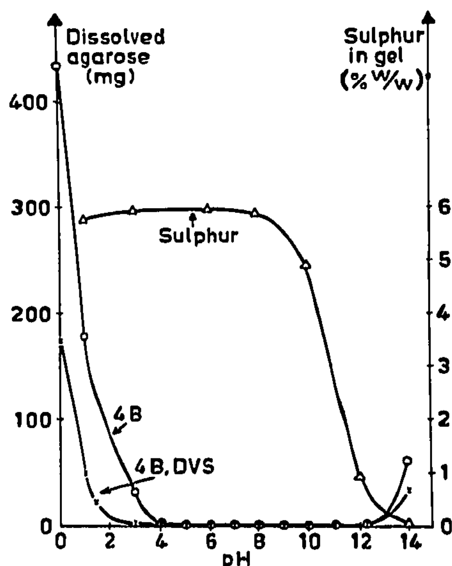


Fig. 4. Gel stability as a function of pH. Sepharose 4B cross-linked with 3% of DVS, and the original Sepharose 4B, were stored at different pH values for 7 weeks. The amount of agarose dissolved from 20 g of the wet gels ($\simeq$450 mg of agarose) was determined. After 21 weeks, the gels were analyzed for sulphur. The following buffer solutions were used: pH $\simeq$0, 1 *M* HCl; pH 1, 1 *M* HCl + 1 *M* sodium acetate; pH 3–5, 1 *M* acetic acid/sodium acetate; pH 5–8, 1 *M* sodium phosphate buffers; pH 9–12, 1 *M* sodium bicarbonate/carbonate buffers; pH $\approx$14, 1 *M* NaOH. The buffer solutions of pH from 3 to 12 contained 0.02% of NaN_3 to prevent bacterial growth.

Chemical stability

Sepharose 4B cross-linked with 3% of DVS and untreated Sepharose 4B were washed with water on glass filters. To portions of 10 g of each wet gel were added 100 ml of 1 *M* buffer solutions of various pH values (see legend to Fig. 4). The gels were stored in stoppered bottles at room temperature (23–25°) for 7 weeks, and the supernatant solutions were analyzed for carbohydrate. After a further 14 weeks of storage, some of the gels were analyzed for sulphur. Carbohydrate analysis in this second series gave essentially the same results as before.

As seen in Fig. 4, elimination of sulphur has already started at a pH slightly above 8. It should be remembered that this decrease is the result of a very long contact time, and that the conditions required for adsorption or desorption of the desired substances are seldom likely to be harsh enough to prevent the use of DVS-agarose. However, it is known that alkylsulphones are split in alkaline media to form sulphinic acids[6] according to the reaction:

$$R{-}CH_2{-}CH_2{-}SO_2{-}R' + OH^- \rightarrow R{-}CH = CH_2 + R'{-}SO_2^- + H_2O \qquad (2)$$

Formation of matrix-bound sulphinate would considerably hamper the use of DVS-agarose, and the following experiment was therefore made to see if the gel was converted into an ion exchanger in alkaline solution.

Sepharose 2B cross-linked with 5% of DVS was divided into portions of 10 g.

Ten milliliters of 0.2 *M* sodium hydroxide were added to each portion, and the mixtures were shaken in a water bath at 50°. Samples were withdrawn at intervals, neutralized and washed with cold water on a glass filter, then analyzed for sulphur, vinyl groups and cation-exchange capacity. As can be seen in Fig. 5, the sulphur content decreases rapidly (about 10% in the first hour), and at this high alkalinity vinyl groups are also eliminated; the concentration of acidic groups, however, remains constant.

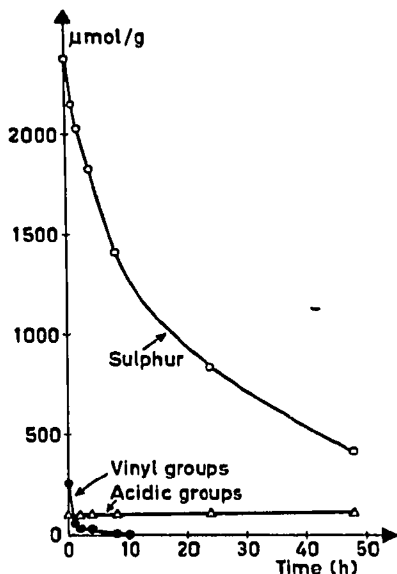
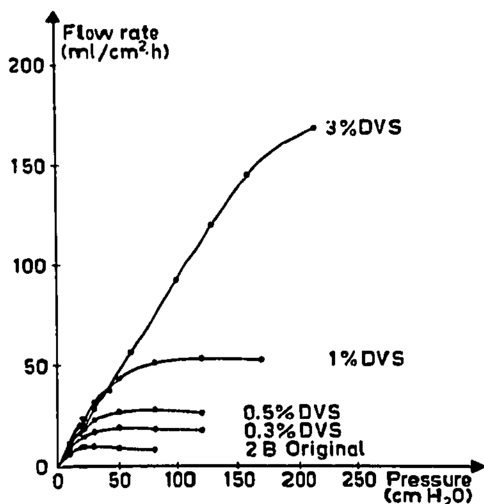


Fig. 5. Effect of alkali on cross-linked gels. Sepharose 2B cross-linked with 5% of DVS (10 g wet weight) was incubated in 0.1 *M* NaOH at 50° in stoppered bottles. At intervals, samples were withdrawn and the amounts of sulphur, vinyl groups and acidic groups were determined. All values were normalized to give the number of μmoles per g of dry gel.

Mechanical stability (rigidity)

The effects of cross-linkage on the rigidity will be most strikingly demonstrated on gels of low matrix densities. Sepharose 2B and 0.5% agarose gels were cross-linked with various amounts of DVS, and columns of identical dimensions were prepared from the gels; flow-rates for such columns were measured at different hydrostatic pressures. A striking improvement in bed stability was noticed as a result of reaction with low concentrations of DVS. After cross-linking with 2 and 5% of DVS, 0.5% agarose could be packed in beds giving flow-rates high enough for chromatography. The flow-rate curves for various gels are shown in Figs. 6 and 7.

Flow-rate change upon divinylsulphonation of Sephadex G-200. The softness of gels sets an upper limit for the practical use of cross-linked dextran as a molecular sieve. Therefore, possible mechanical stabilization with DVS might extend the useful permeation range of dextran gels. To find out if this was so, 200 ml of Sephadex G-200 were cross-linked with 5% of DVS. A marked increase in flow-rate was noticed, but, at the same time, the gel volume had decreased to 72 ml. The Sephadex type most closely resembling the DVS-gel is Sephadex G-75. Flow-rate profiles were determined in two



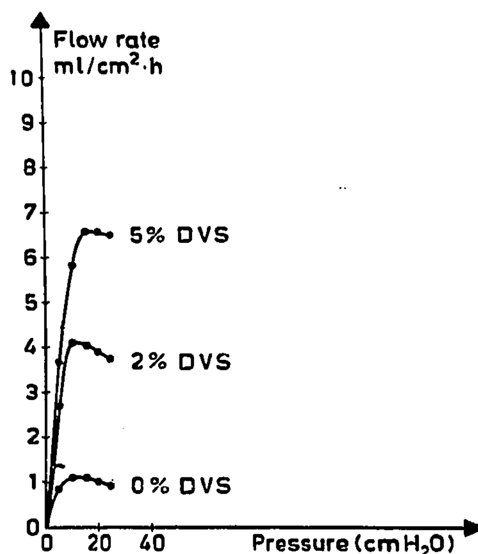


Fig. 6. Mechanical rigidity of cross-linked Sepharose 2B. Cross-linked gels (200 ml) were packed in beds of approximate dimensions 2.5 cm × 40 cm and the flow-rate was measured at various hydrostatic pressures. Experimental details in text.

Fig. 7. Mechanical rigidity of cross-linked 0.5% agarose beads. Flow-rate profiles of 0.5% agarose beads cross-linked with various amounts of DVS. The experiment was performed as in Fig. 6. The beads were prepared in the laboratory from alkali-treated agarose and sieved between 45 and 250 μm before use.

beds of identical dimensions (*ca.* 2.5 × 14 cm; V_t = 72 ml), one filled with 5% DVS-Sephadex G-200, and the other with Sephadex G-75. It can be seen in Fig. 8 that the two curves were very similar. The DVS-Sephadex contained 6.33% of sulphur.

The sulphone group and gel rigidity. In one experiment, with the aim of elucidating the possible role of the sulphone groups in effecting increased rigidity, Sepharose 2B was reacted with 3,4-epoxytetrahydrothiophene 1,1-dioxide in alkaline medium. The product contained 0.25% of sulphur, *i.e.*, it had approximately the same sulphone content as Sepharose 2B cross-linked with 0.1–0.2% of DVS. The rigidity, as determined by flow-rate measurements, was not improved.

Chain length of cross-linking agent and gel rigidity. Since the sulphone groups do not affect the rigidity of DVS-agarose gels, the only possible cause must be related to the lengths of the bridges interconnecting the carbohydrate chains. A series of dibasic acid chlorides was used to study this factor; the results of the study are shown in Table I, from which it can be seen that, although a difference in the degree of substitution (cross-linking) may be of some importance, there is no doubt that gel rigidity is strongly dependent on the inter-carbonyl distance.

Chromatographic tests

Although, with Sephadex, the DVS reaction results in strong contraction, the agarose gels do not seem to change their volume as a consequence of cross-linking. It is therefore likely that the permeation behaviour of the agarose gels is not drastically altered by the DVS treatment.

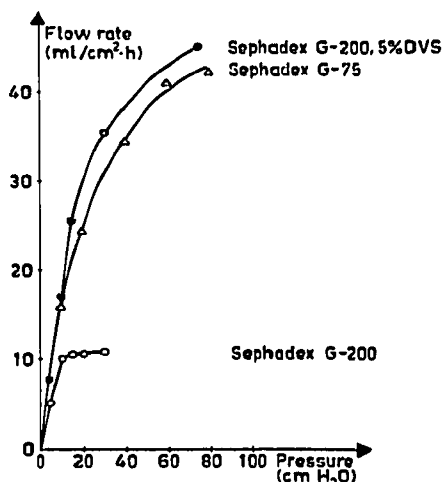


Fig. 8. Flow-rate change after treatment of Sephadex G-200 with DVS. The flow-rate profile of 200 ml of Sephadex G-200 was determined. The gel was collected, treated with $NaBH_4$ overnight to increase the stability to alkali and cross-linked with 5% of DVS. The gel volume shrunk to 72 ml, hence the flow-rate of the cross-linked gel was compared to that of 72 ml of Sephadex G-75 (the most similar Sephadex gel).

Blue Dextran 2000, human serum albumin and thyroglobulin (1 mg of each dissolved in 1 ml of 0.05 *M* Tris hydrochloride of pH 7.5) were separately run on the same column of Sepharose 6B (1.5 × 70 cm; V_t = 122 ml). The flow-rate was kept at 7.5 ml/h and 1.8-ml fractions were collected. The gel was then removed quantitatively and cross-linked with 5% of DVS. The settled volume of the DVS-gel was 130 ml; thus, there was a slight increase in bed-volume. From this gel, 122 ml were packed in the same tube as before and the same chromatographic runs were repeated; the results are shown in Table II.

Sepharose 2B and 1% DVS-Sepharose 2B were packed into beds of equal dimensions (1.6 cm × 91.2 cm; V_t = 183 ml) in 0.05 *M* Tris hydrochloride of pH 7.5.

TABLE I

PROPERTIES OF SEPHAROSE 2B CROSS-LINKED WITH DIBASIC ACID CHLORIDES

Cross-linking agent	*Length of cross-linking bridge,*		*Wet weight of gel after cross-linking (g)*	*Max. flow-rate** (ml/cm²·h)*
	atoms	*Å**		
Succinic acid chloride	4	4.6	112	50
Glutaric acid chloride	5	6.2	159	300
Adipic acid chloride	6	7.7	180	80
Terephthalic acid chloride	6	7.3	180	90
Divinyl sulphone	5	6.7	210	10–200***
Before cross-linking	—	—	200	10

* Based on the following atomic distances: C–C, 1.54 Å; C–S, 1.82 Å; C–C (benzene), 1.40 Å.
** Approximate values, only relevant to the experimental conditions given in the text.
*** Depending on the concentration of DVS, see Fig. 6.

TABLE II

CHROMATOGRAPHY OF MODEL SUBSTANCES ON SEPHAROSE 6B AND 5% DVS-SEPHAROSE 6B

Substance	*Sepharose 6B*		*DVS-Sepharose 6B*	
	V_e *(ml)*	K_{av}	V_e *(ml)*	K_{av}
Blue Dextran 2000 (V_0)	39.5	0.00	49.1	0.00
Human serum albumin	79.5	0.48	91.5	0.58
Thyroglobulin	57.3	0.22	74.4	0.35

Chromatographic experiments were then performed with 1.0 ml samples of TMV (particle weight 40 × 10^6 daltons), Blue Dextran 2000 (MW about 2 × 10^6 daltons), thyroglobulin (MW 670,000 daltons) and human serum albumin (MW 68,500 daltons). The flow-rate was kept at 8 ml per h, and 2.65-ml fractions were collected. A composite chromatogram for the DVS-column is shown in Fig. 9; almost identical chromatograms were obtained with Sepharose 2B.

Chromatographic experiments were also performed on 0.5% agarose cross-linked with DVS in concentrations of 2 and 5%; the original non-cross-linked gel was too soft to be tested. The column dimensions were 1.5 cm × 83.5 cm (V_t = 131 ml), the buffer of pH 8 contained 0.01 *M* Tris, 0.003 *M* succinic acid, 0.01 *M* magnesium chloride and 0.42 ml of 2-mercaptoethanol per litre, the flow-rate was 2–4 ml per h and the fraction volume was 2.5–3.5 ml. The test substances were dissolved in 1 ml of buffer and run separately on the columns. All experiments were performed at 5°. Owing to slight compression of the beds, it was not possible to achieve steady flow-rates and constant fraction volumes. The chromatographic patterns obtained were practically

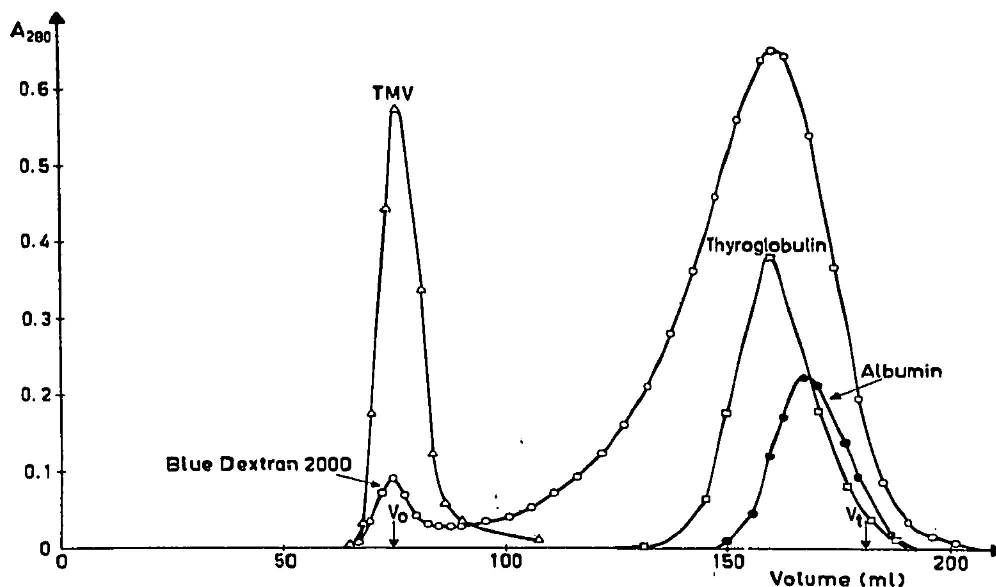


Fig. 9. Chromatography of model substances on Sepharose 2B cross-linked with 1% of DVS. Experimental data in text.

identical. Fig. 10 shows superimposed chromatograms from the separate runs with the model substances on 5% DVS–0.5% agarose. All substances except the ribosomes were eluted with 100% recovery. The yields of ribosomes were 51 and 33% on the 2% and 5% DVS-agarose, respectively. The corresponding ribosome yields from similar experiments with non-cross-linked Sepharose 2B and 4B were 31 and 35%, respectively. In all experiments, an additional 30% of the applied amount of ribosomes could be displaced from the columns with *M* sodium chloride.

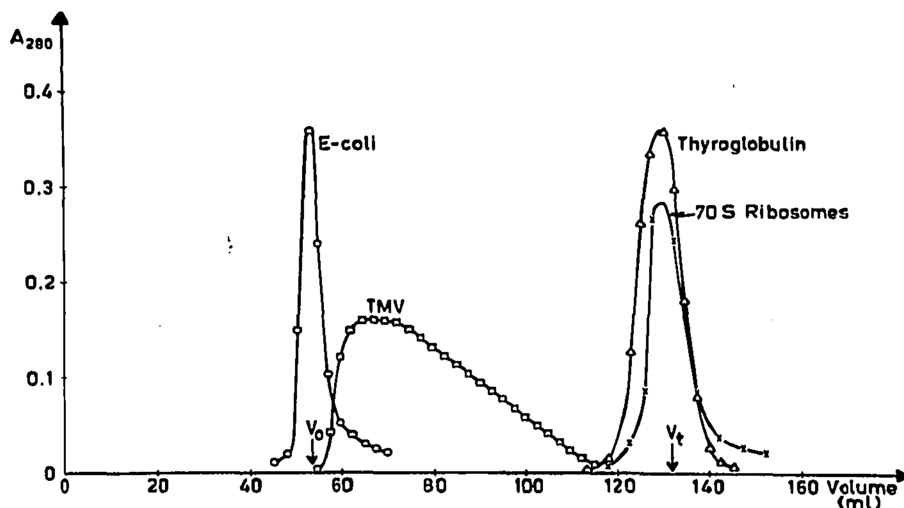
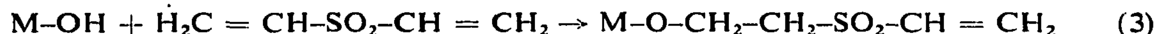


Fig. 10. Chromatography of artificial samples on 0.5% agarose cross-linked with 5% of DVS. Experimental data in text.

DISCUSSION

DVS has long been used as a cross-linking agent[7]. Shortly after the introduction of epichlorohydrin cross-linked dextran (Sephadex)[8], Flodin[9] prepared very dense gels by reaction of Sephadex G-25 with DVS. These gels were also studied by Marsden[10] and one of us[11]; they showed no unexpected properties. Also, the study made by us does not reveal anything unexpected for DVS-Sephadex G-200. The reaction with agarose, however, gives a product of considerable interest. DVS-agarose approaches the ideal gel matrix for a molecular sieve, for adsorbents for bio-affinity chromatography, and for immobilized enzymes in the pH range used in most applications. It is therefore important to learn more about the reaction and the agarose products.

The first step in the reaction is the following:



$$M\text{-}OH + H_2C = CH\text{-}SO_2\text{-}CH = CH_2 \rightarrow M\text{-}O\text{-}CH_2\text{-}CH_2\text{-}SO_2\text{-}CH = CH_2 \quad (3)$$

where M signifies the matrix.

Further reaction between the reactive vinyl side group and another hydroxyl in the matrix yields a cross-link:

$$M\text{-}O\text{-}CH_2\text{-}CH_2\text{-}SO_2\text{-}CH_2\text{-}CH_2\text{-}O\text{-}M \quad (4)$$

The reaction is slow at pH 9 and much faster at pH 11, as can be inferred from the results shown in Fig. 1. Further, it can be seen from Fig. 2 that the reaction is much slower at 0° than at (or above) room temperature, and many hours are required for conversion of the gel into a form insoluble in hot water.

The cross-linked gels prepared by reaction with 3 and 5% of DVS for 2 h contain 6–8% of sulphur, *i.e.*, 22–30% cross-links.

There is a slow breakdown of cross-links in alkaline media, as shown by the decrease in sulphur content (Fig. 4), the concomitant softening of the gel and the increasing solubility. This undesired reaction is very weak, but noticeable after long contact times (weeks or months) at pH 9; it is considerably accelerated at higher pH and elevated temperature. However, it is slow compared to the cross-linking reaction. Since no sulphinic acid groups (reaction 2) can be found in the gel after storage in alkaline buffers, it is clear that ether cleavage occurs much faster and at lower pH than oxidation reactions involving C–S bonds. The hydrolytic cleavage of the bis-ethylenesulphone bridges must take place in the following manner:

$$M\text{-}O\text{-}CH_2\text{-}CH_2\text{-}SO_2\text{-}CH_2\text{-}CH_2\text{-}O\text{-}M \xrightarrow{+H_2O} M\text{-}O\text{-}CH_2\text{-}CH_2\text{-}SO_2\text{-}CH_2\text{-}CH_2\text{-}OH + HO\text{-}M \quad (5)$$

$$M\text{-}O\text{-}CH_2\text{-}CH_2\text{-}SO_2\text{-}CH_2\text{-}CH_2\text{-}O\text{-}M \longrightarrow M\text{-}O\text{-}CH_2\text{-}CH_2\text{-}SO_2\text{-}CH = CH_2 + HO\text{-}M \quad (6)$$

Since no increase in vinyl groups could be found, the first alternative must be valid. In the next step, bis-(2-hydroxyethyl) sulphone (never isolated) is presumably formed, whereby sulphur is removed from the matrix.

The cross-linking of a gel is expected to cause volume changes. To judge from the K_{av} values obtained for 5% DVS-Sepharose 6B (Table I), the reaction with DVS gave a product of considerably increased porosity. It is also possible that the increase in K_{av} may to some extent be caused by adsorption, since we have noticed that the DVS-gels show some hydrophobic interaction with proteins. By using the chromatographic data, the volume of beads was calculated. Surprisingly, the gel seems to have contracted by about 5%, although the volume of settled grains had actually increased. These volume changes indicate that the beads have become stiffer and less prone to deformation.

The rigidity increase makes it possible to apply much higher pressures to the beds and so to increase flow-rates; the practical consequences of this are obvious. Chromatography based on molecular sieving can be extended to a higher molecular size range. It should be possible to fractionate viruses, subcellular particles and membrane fragments in agarose beds of very low matrix content. When necessary for achieving particularly good separations, beds packed with smaller beads (leading to lower theoretical plate heights) may be preferred.

Finally, it should be possible to increase the bed dimensions both in height and width, a prerequisite for technical applications of molecular sieving, affinity chromatography and immobilized enzymes.

The increase in rigidity without concomitant decrease in permeability as a result

of reaction with DVS is a characteristic feature for agarose gels not shared with Sephadex and polyvinyl alcohol (first otherwise cross-linked). Therefore, it is evident that the properties of the DVS-agarose gels are determined by the structure of the agarose as well as that of the cross-linking agent. Since reaction with 3,4-epoxytetrahydrothiophene 1,1-dioxide does not significantly alter the gel rigidity, the introduction of sulphone groups should not be the important factor responsible for this behaviour. However, because of their polar character, they may render the gels somewhat more hydrophilic than would otherwise be the case. Instead, the important molecular parameter is the interatomic distance between the terminal carbon atoms in the vinyl groups. From this postulate, we may conclude that any bifunctional reagent with the same (or almost the same) distance between the reacting groups will convert agarose gels into gels of much higher mechanical strength. This is corroborated by the study made with the dibasic acid dichlorides.

We interpret our findings in the following way. There are large "empty" regions or "caves" in an agarose gel due to heterogeneous matrix distribution. These "caves" are lined by "pallisades" or a comparatively dense mesh of carbohydrate chains having a restricted motility. The oscillations in relative positions of the polysaccharide chains impart flexibility to the gel, with a resulting decrease in rigidity. DVS, glutaric acid dichloride and to a lesser extent succinic, adipic and terephthalic acid dichlorides are capable of locking or tying the chains together and thereby decreasing the amplitude of the oscillations.

The dense matrix regions contain double helices arranged in bundles as found by Rees and his coworkers in κ- and ι- gel-forming types of carrageenans[12]. The stabilizing cross-links may be either intra- or inter-helical (see Fig. 11). Of course, stabilizing cross-links may also be formed in non-helical regions, but this would not account for the strong dependence on the length of the cross-linking agent referred to above.

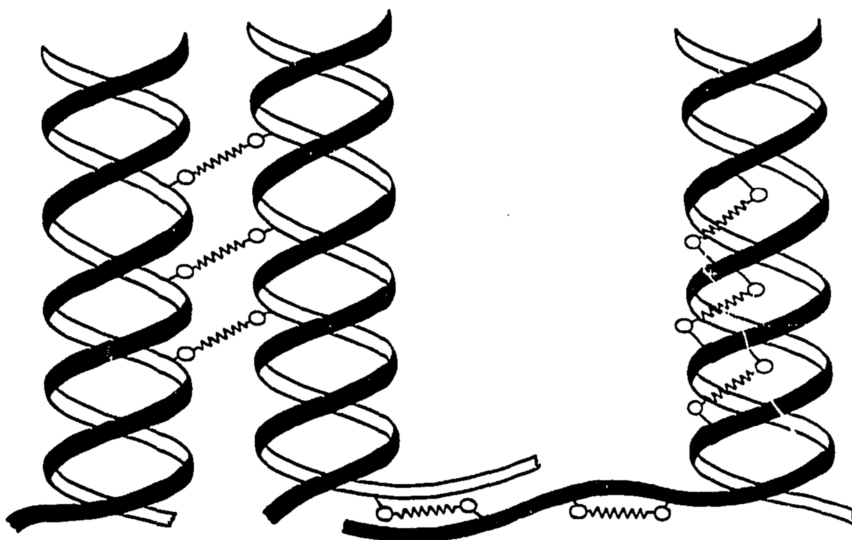


Fig. 11. Cross-linked agarose gels. The cross-links (symbolized by zig-zag line) can occur as intra- or inter-molecular bridges in helical or non-helical regions in the gel. The figure shows schematically the different possibilities.

Since helical bundle structures hardly exist in the hydrogels of the synthetic polymers of today, it seems unlikely to us that the latter should be able to replace suitably cross-linked agarose as matrices for affinity adsorbents or for immobilization of enzymes, as the high hydrophilicity, permeability and high mechanical strength seems to require reinforced multi-strand network structures. The only limitations of significance remaining for the agarose derivatives are chemical instability in acid (and, sometimes, basic) regions of pH and the presence of minute amounts of adsorption centres (ionic and hydrophobic groups). For most biochemical applications, these limitations are of no importance. For technical applications, economic considerations might provoke new technology for the production of rigid agar or agarose gels.

ACKNOWLEDGMENTS

We thank Birgitta Larsson and Ingmar Olsson for their valuable technical assistance.

The work was supported by The Swedish Board for Technical Development.

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