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REVERSE SALT GRADIENT CHROMATOGRAPHY OF tRNA ON UNSUBSTITUTED AGAROSE

III. PHYSICAL AND CHEMICAL PROPERTIES OF DIFFERENT BATCHES OF SEPHAROSE 4B

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SUMMARY

Four batches of Sepharose 4B, which gave differing elution profiles in reverse salt gradient chromatography of tRNA, were analysed by a number of techniques. Charged groups were estimated from infrared spectra, and gave 0.04–0.05 sulphates and 0.18–0.27 carboxyls per disaccharide. Nuclear magnetic resonance showed the presence of 0.21–0.23 6-O-methyl substituents per disaccharide. The mass concentrations of the beads in aqueous suspension (including agarose-bound water) ranged from 4.7 to 5.0%. High-resolution scanning electron microscopy demonstrated a three-dimensional network of threads with diameters between 2 and 20 nm. None of these properties varied appreciably between batches. There was, however, a variation in the size distribution of beads. In two batches which induced a high degree of adsorptive retardation during chromatography, very few beads exceeded 100 μm in diameter; two other batches which gave inferior retardation had a preponderance of somewhat larger beads. The significance of this observation remains unclear.

INTRODUCTION

In previous papers[1,2] it was established that two mechanisms operate in the reverse salt gradient chromatography of tRNA on Sepharose 4B. One mechanism involves interfacial precipitation, and varies with conditions in the same way as the solubility of the given tRNA in free solution. The other appears to be largely independent of the source of tRNA and to be very similar in magnitude at temperatures of 5°C and 25°C. It was suggested[2] that this mechanism, which induces adsorptive retardation of molecules after release from interfacial precipitate, involves the formation of a monolayer on the threads of the agarose network. The binding involved is probably analogous to "hydrophobic bonding".

Since adsorptive retardation was found to be subject to considerable variation between batches of Sepharose[1], a number of physical and chemical properties were measured in the hope of establishing a correlation with the variation in binding

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behaviour. The present paper reports these measurements, which are of intrinsic interest because some of the techniques have not previously been applied to Sepharose. The remaining uncertainty about the source of the batch variation is also discussed.

MATERIALS AND METHODS

tRNA was as used earlier[1,2], and batches of Sepharose 4B as specified in Table I of Part I[1]. Blue Dextran 2000 (lot No. 2678) was supplied by Pharmacia.

Infrared (IR) spectra were recorded on a Grubb–Parsons Spectromaster and replotted as absorbance against wavenumber. Samples were prepared from desalted Sepharose dissolved in boiling water and dried under vacuum on calcium fluoride plates. A similar plate was inserted into the reference beam.

Nuclear magnetic resonance (NMR) measurements were carried out by Messrs. D. Welti and S. M. Bociek of the Colworth Laboratory of Unilever Research, using a Bruker WP200 spectrometer. Material in aqueous suspension was first desalted by flushing with water in a small column. Samples in ^2H_2O were studied at 95°C, using 200.1 MHz for ^1H and 50.32 MHz for ^{13}C. Spectral peaks were identified by comparison with those reported for ^1H at 300 MHz (ref. 3) and for ^{13}C at 67.9 MHz (ref. 4). Peak areas were measured from expanded plots by cutting out and weighing the peaks.

Interference microscopy was carried out on a Zeiss WL microscope equipped with Smith–Lebedeff interferometer optics and a calcite Ehringhaus compensator. Material in aqueous suspension was first desalted as above.

Scanning electron microscopy was carried out on a JEOL Model JEM-200CX in the SEI mode, operated at 200 kV with the "spot size" control activated to reduce beam damage. Specimens were resuspended in acetone by sequential centrifugation through graded mixtures of acetone and water. They were then subjected to critical-point drying with carbon dioxide in apparatus E3000 supplied by Polaron (Watford, Great Britain). It was found advisable to allow 3 h for liquid exchange between the two periods of flushing with carbon dioxide. Beads were then sputter-coated with gold for 1 min at 10 mA at a distance of 40 mm from the cathode of apparatus SCD020 (Balzers, Balzers, Liechtenstein), filled with argon at a pressure of *ca.* 0.05 mbar. To ensure a reproducible thickness of coating it was necessary to monitor the absorbance of the deposit on a piece of filter paper: the first deposit obtained after the cathode had been exposed to air for some time was often less than those obtained by repeating the process.

Bead size distributions were obtained by measurement of photographs taken of aqueous suspensions of Sepharose, using bright-field light microscopy.

RESULTS AND DISCUSSION

Estimation of charged groups

Although agarose contains a lower proportion of charged residues than the agar from which it is purified, residual charges remain which are attributed to sulphate and carboxyl groups (including pyruvate)[5,6]. It was initially hoped that these could be estimated by direct titration. Material was first desalted by exhaustive flush-

ing with water distilled after passage through a deionizer. Subsequent addition of salt led to a fall in pH due to release of protons from the agarose. However, the charge concentrations calculated from these experiments were all much lower than those deduced by the second method described below, and it was concluded that the groups were probably not fully protonated under the conditions used.

The second method involved the recording of IR spectra. Sulphate esters of carbohydrates show a strong S = O stretching vibration at 1240 cm^{-1}, though there are many overlapping bands in this region[7]. It is also possible, with samples dried from different pH values, to estimate carboxylic and pyruvic acid residues; when protonated these give a C = O stretch vibration at 1725 cm^{-1}.

Fig. 1 illustrates a typical pair of spectra from one batch of Sepharose. There is only a small peak at *ca.* 1250 cm^{-1}, but a much larger one at 1725 cm^{-1} which is present only at acid pH.

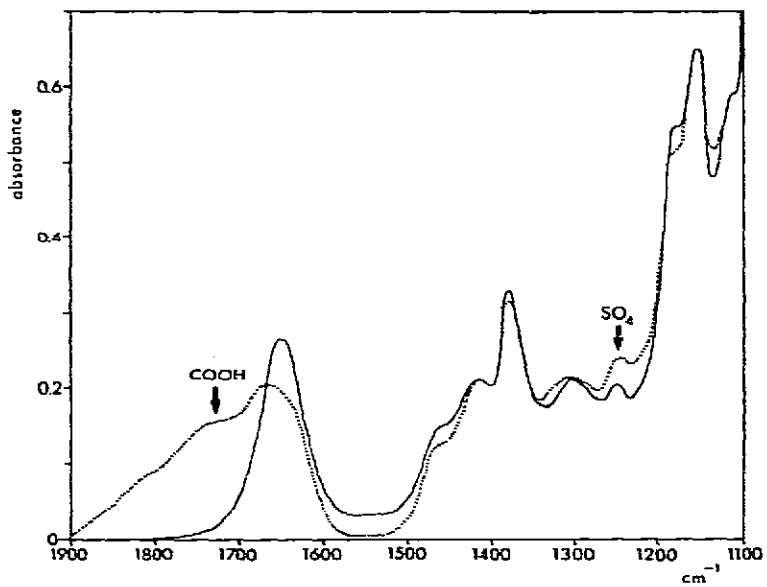

Fig. 1. IR spectra of Sepharose 4B (batch E). Dotted line, pH 1.5; solid line, pH 7.

To calculate an upper limit to the sulphate content, the peak height at *ca.* 1250 cm^{-1} was estimated by assuming a background contribution from neighbouring peaks, and comparing with the absolute height of the peak at 1155 cm^{-1} which arises from the antisymmetric bridge oxygen stretching vibration. The ratio thus obtained was compared with that calculated from the spectrum of alginic acid containing three sulphate residues per disaccharide[7,8]. For carboxyl content, the difference between spectra at pH 1.5 and 7.0 gave a peak at 1725 cm^{-1} which was compared with that shown by hyaluronic acid having one carboxyl residue per disaccharide[7,8]. The absorbance ratio with the peak at 1155 cm^{-1} was again used to normalize the spectra.

Results are summarized in Table I. Sulphate values appeared higher than that obtained by Hjertén[6] from direct sulphur analysis of purified agarose (0.14%, w/w, or 0.013 sulphates per disaccharide), but they were in any case very similar for all

TABLE I

PROPERTIES OF SEPHAROSE 4B

Batch	*No. of groups per disaccharide (mol. wt. 306)*			*Mass concentration of beads (%)*
	*Sulphate**	*Carboxyl**	*O-Methyl***	
C	0.04	0.27	0.21	5.0
D	0.04	0.21	0.22	4.8
E	0.05	0.26	0.23	4.8
F	0.04	0.18	0.22	4.7

* By IR spectroscopy; ±0.03.
** By NMR spectroscopy; ±0.01.

batches; there was no variation that could explain the difference in fractionation behaviour[1] between Sepharose batches C and E. Carboxyl values were also similar for these two batches. Total calculated charge contents were, for all batches, *ca.* 0.3 per disaccharide or 1 mequiv. g^{-1}. Since a packed column of wet Sepharose 4B contains about 3% agarose, this means a charge content of *ca.* 0.03 equiv. l^{-1}.

Ion-exchange resins commonly show capacities of the order of 1 equiv. l^{-1}. Thus the values for Sepharose, though much lower, are by no means negligible. For the monolayer of tRNA discussed in the previous paper[2], about twenty charges would be in the vicinity of each tRNA binding site. Against this must be set the fact that the high salt concentrations used will tend to shield the charges. The charges are of course negative, so they would not be expected to contribute to a positive attraction of tRNA to Sepharose. They could, however, modify the effect of some other mechanism. To explain the batch variation in fractionation behaviour, the charges would need to vary in their *distribution* along the threads, but there is no evidence on this point.

Methylation of agarose

NMR spectra showed no major differences between batches. ^{13}C Spectra (not illustrated) were largely as expected for pure agarose[4], but ^{1}H spectra (Fig. 2) indicated appreciable 6-O-methyl substitution[3]. Values are tabulated in Table I. Although this again did not explain the variations in chromatographic behaviour, such methylation must be presumed to contribute to "hydrophobic bonding" between solutes and agarose.

Concentration measurements

Interference microscopy was used both to check the concentration of agarose in the beads of different batches, and to monitor specimen changes during preparation for electron microscopy. In order to convert optical path differences into concentrations it was first necessary to determine specific refractive increments in both water and air. These were obtained indirectly from refractive index and density measurements, using the formula for the increment $\alpha = (n - n_m)/100\varrho$; n and n_m are the refractive indices of suspended solid and liquid medium respectively, and ϱ is the density of the solid. Material was prepared by critical-point drying (see Materials and

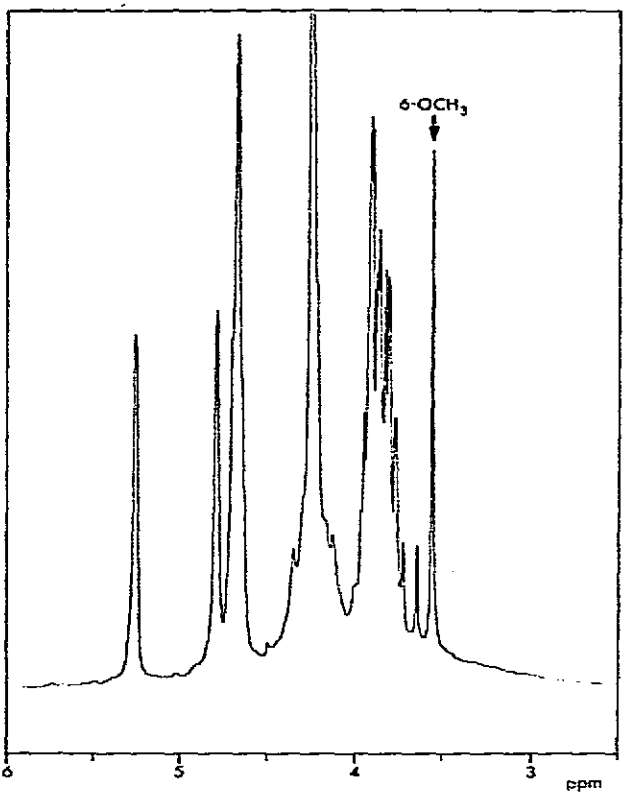

Fig. 2. ^{1}H NMR spectrum of Sepharose 4B (batch E). Number of scans, 200. Shifts are relative to an arbitrary reference point as no internal standard was used.

methods) and n determined to be 1.54 by resuspension of both dry and acetone-suspended beads in a range of oils of known refractive index, ϱ was determined to be 1.36 by flotation of beads in mixtures of acetone and F113 (1,1,2-trichloro-1,2,2-trifluoroethane). The rather low density value suggests that preparation by the method used does not remove bound water, which is believed to lie in the central cavity of each agarose helix[9]. Calculation then gives $\alpha = 1.5 \cdot 10^{-3}$ for a 1% concentration change in water, and $\alpha = 4.0 \cdot 10^{-3}$ in air.

For each batch of Sepharose a range of bead sizes was examined, and for each bead the diameter and optical path difference at the centre were recorded. Assuming the thickness at the centre to be equal to the measured diameter, the optical path difference, Δ, was then plotted against diameter, d, to give a straight line (Fig. 3) whose slope was determined. The percentage concentration c was obtained from the equation $c = \Delta/\alpha d$. Table I shows that for untreated Sepharose beads in water, c was in all cases between 4.7 and 5.0%. These values include the bound water. The minor variations in c did not correlate with variations in fractionation behaviour.

Scanning electron microscopy

Fig. 4 illustrates high-resolution scanning electron microscopy of the surface of Sepharose beads, after critical-point drying. Interference microscopy after drying

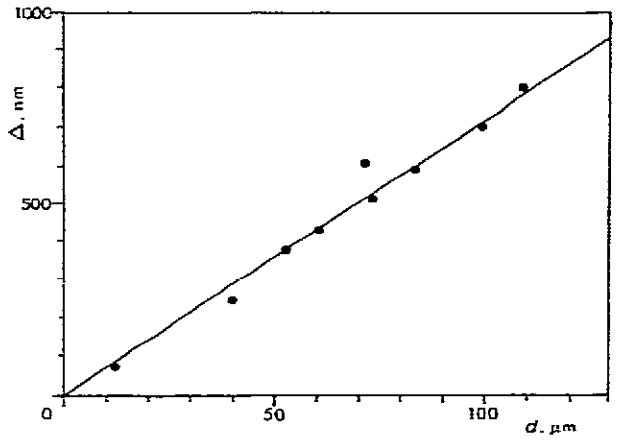

Fig. 3. Plot of optical path difference at 546 nm against bead diameter, for an aqueous suspension of Sepharose 4B (batch E).

indicated concentration increases consistent with a dimensional shrinkage of *ca.* 20%; on the other hand, conventional freeze-drying produced gross distortions.

The thickness of the gold coating was estimated from separate experiments (not illustrated) in which the time of sputtering was varied and the average thread diameter measured; as a further check, coatings were deposited on glass and their thickness measured by multiple-beam interferometry[10]. For Fig. 4 the coating was calculated to have a thickness of 3 nm, giving an increase of up to 6 nm in the apparent thickness of each thread. The apparent thicknesses ranged between *ca.* 8 and 26 nm, so that the bundles of agarose helices had diameters between 2 and 20 nm. These values are consistent with observations by others[11] of stained and sectioned

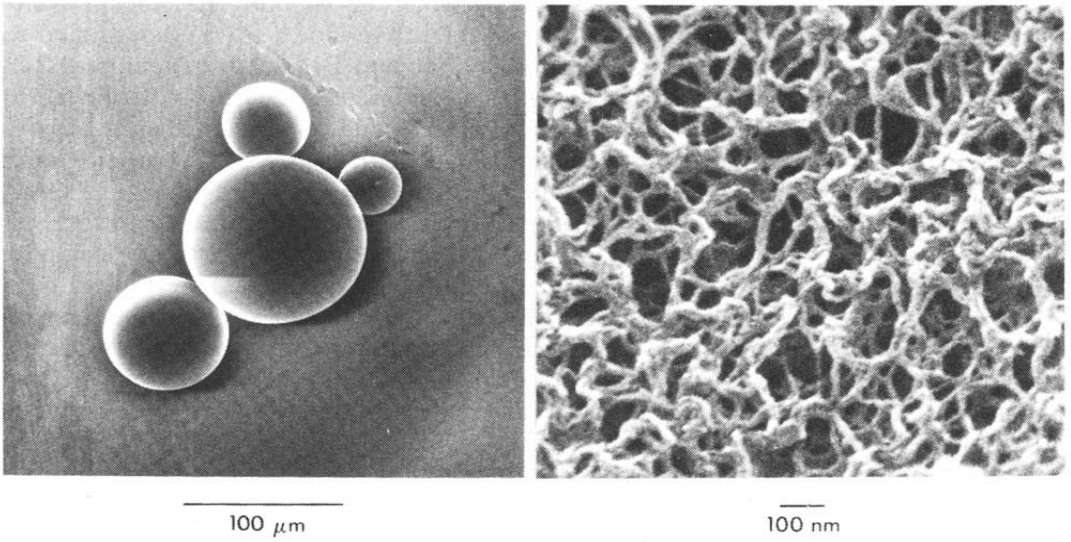

Fig. 4. Scanning electron microscopy of Sepharose 4B (batch E).

beads. There was no obvious variation between batches, nor between large and small beads.

Bead size distributions

Fig. 5 illustrates histograms of bead size distribution, representing the percentage of column *volume* occupied by beads of different diameter. It is apparent that batches E and F differed from C and D, in that they contained a higher proportion of beads with diameter greater than 100 μm. Batch E showed the largest spread in diameters of all the batches. It was previously established that isocratic fractionation profiles of batches C and D were similar, showing the multiple peaks illustrated in Fig. 7c of Part I[1]. In contrast, batch E showed reduced retardation and inferior resolution of the kind illustrated in Fig. 7d of Part I. Batch F (not illustrated) gave intermediate behaviour, with poor resolution but fairly high retardation.

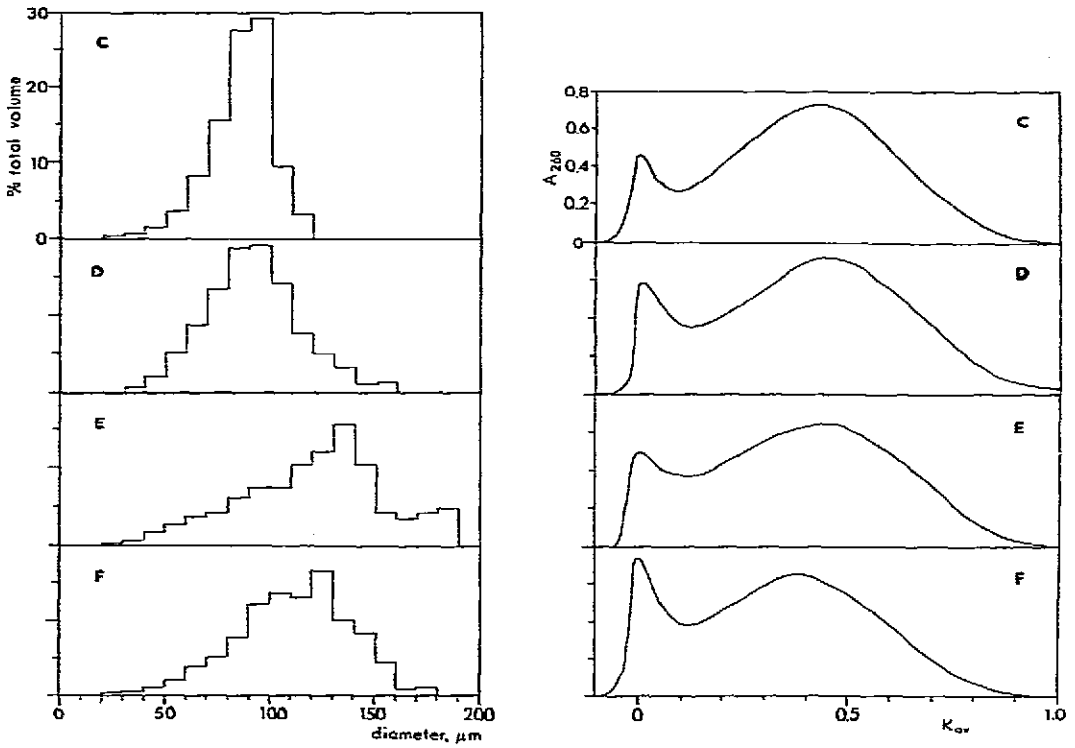

Fig. 5. Size distribution of beads in Sepharose 4B. The letter by each histogram denotes the batch studied. For each range of diameters, the percentage of total volume occupied by the beads is plotted as ordinate. The number of beads measured for each histogram was about 700.

Fig. 6. Blue Dextran 2000 elution profiles. The letters indicate the batch of Sepharose 4B used. In each case a small aliquot of 0.3% blue dextran was applied to a column previously equilibrated at 5°C with 1.3 *M* ammonium sulphate, 10 m*M* magnesium chloride, 1 m*M* EDTA and 10 m*M* sodium phosphate buffer, pH 4.5. Elution was carried out at 15 ml h^{-1} using the same solution. Column diameters were 16 mm and the bed lengths 775, 555, 520 and 830 mm for batches C, D, E and F, respectively. K_{av} is defined as $(V_e - V_0)/(V_t - V_0)$ where V_t = total bed volume, V_0 = excluded (void) volume and V_e = eluted volume. For the four experiments illustrated, V_t/V_0 (a measure of the tightness of packing) had values of 2.9, 2.8, 2.7 and 2.7.

One can only at present speculate on the meaning of these results. In the absence of significant differences in chemical composition or small-scale structure, one might suggest an effect related to the rate of depth of penetration of tRNA into the beads. However, an experiment in which tRNA was stained immediately after binding to a column bed (not illustrated) gave no support to this idea; tRNA seemed uniformly distributed throughout the beads. One would not, therefore, expect a variation of resolution with particle size of the kind experienced when molecules are adsorbed to the surface of solid beads. We have already seen in Part II[2] that the binding data are consistent with the formation of a monolayer of tRNA on the whole of the agarose matrix; the external surface of each bead would offer only a small fraction of the total agarose surface.

Pore size distributions

As a semi-quantitative test both of the exclusion limit and the pore size distribution, Blue Dextran 2000 was applied to the four batches of Sepharose listed in Table I. Results are illustrated in Fig. 6. The manufacturers state that blue dextran has an average molecular weight of $2 \cdot 10^6$, while the exclusion limit for Sepharose 4B is $5 \cdot 10^6$. The peaks in Fig. 6 at $K_{av} = 0$ show that some of the blue dextran exceeded this limit, while most of it ran in a broad peak centred on $K_{av} = 0.4$. Although the four profiles are not identical, no single feature seems to correlate with the batch variation in adsorptive retardation; although the broad peak for batch F has a slightly smaller K_{av} than in the case of batches C and D, batch E does not show the same effect.

CONCLUSION

A further exploration of batch variations will be needed to decide whether the bead size distribution is as relevant as the present results suggest, or whether the correlation is fortuitous. It might also be useful to separate beads in different size ranges from a given batch, and to test each range separately by the techniques described in this series of papers. For the samples studied in the present work there was unfortunately an insufficient quantity to make this feasible. The variation in adsorptive retardation is evidently not related to any gross change in physical or chemical properties. Something in the manufacturing process may have varied; on the other hand, the batches studied were manufactured over a period during which there was a change in the supplier of raw material (personal communication from Pharmacia).

Until there is a clear-cut explanation of the variations, it will be necessary for users of the reverse-gradient technique to test each batch separately. Although there are large batch variations in adsorptive retardation, Fig. 5 of Part I[1] shows that reasonably satisfactory fractionation of tRNA is possible if a sufficiently extended gradient is employed. However, the best resolution is undoubtedly associated with a high degree of adsorptive retardation, and it is unfortunate that recently manufactured batches (such as E and F) were found to be inferior in this respect. For isocratic work, only the earlier high-retardation material would be suitable.

The results in the present paper indicate that bound charges and methyl substituents may both have a minor influence on the binding properties of Sepharose, since they are present in far from negligible amounts. The scanning electron micros-

copy demonstrates the unusual stability of the three-dimensional agarose structure, provided that drying artifacts are avoided; Sepharose beads might well serve as useful model specimens for the preparative techniques used in studying biological structures such as cytoskeletal networks.

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