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GEL MATRICES FOR SCANNING GEL CHROMATOGRAPHY

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I have examined the light-scattering behavior of a number of gel matrices used in gel filtration chromatography. The angular dependence of light scattering by Sephadexes is consistent with treatment of the particles as large scattering particles with a low refractive index increment (μ). Such particles scatter light almost exclusively in the forward direction, permitting their use in direct scanning gel chromatography systems without corrections for multiple scattering and the consequent variation in pathlength through the column. Any matrix material with an appropriate combination of these two properties (large effective size and low μ relative to solvent) will perform reasonably well in direct scanning systems while any material with very small effective particle size (agaroses) or high μ (glass beads) may be expected to perform poorly. Agaroses may be acceptable for work in the visible region of the spectrum while glass beads are not.

1. Introduction

A decade ago, Brumbaugh and Ackers [1] showed the feasibility of direct scanning gel chromatography, in which the absorbance of a sample is measured while it resides on a gel column, rather than after elution from the column. They clearly established that the absorbance of a solution of myoglobin obeyed Beer's law to a high degree of precision even though there was a loss of more than 90% of input light caused by scattering from the gel matrix.

In a system that shows scattering of light there must be a difference of pathlength between source and receiver for scattered light relative to directly transmitted light. Since the degree of scattering is a function of wavelength, the relative pathlength effect will vary with wavelength also. This is one of the factors that leads to distorted absorption bands in materials with high scattering power such as mitochrondrial suspensions or specimens examined in the frozen state in aqueous media (cf. Butler, 1972 [2]). However, Ackers and coworkers have demonstrated that the scattering of light by the gel matrices they use causes no observable distortion of the absorption spectrum for the materials of interest to them. As mentioned above, Brumbaugh and Ackers [1] showed that the absorption spectrum of myoglobin within a Sephadex G100 gel matrix was highly correlated with the absorption spectrum in free solution above the gel (deviation less than 1%) at a myoglobin absorbancy up to 1A greater than that of the gel. Ackers et al. [3] showed, using photon counting, that the absorbancy of myoglobin at 220 nm is linear up to 4A (more than 2A above the scattering baseline). Thus the pathlength error must be less than 0.1% for a matrix of Sephadex G100.

This apparent paradox can be resolved if one considers that the gel particles are very large scattering particles with a very low refractive index increment above that of the solution phase. This combination leads to scattering of light almost exclusively in the forward direction [4]. Evidence that this is in fact an appropriate way of describing gel matrices is presented in this paper.

2. Materials and methods

Sucrose, Tris, and glycerol were reagent grade; human hemoglobin was from Sigma Chemical Co. (lot #555-8271). Sephadex G-75 regular (lot 5445), G200 regular (lot 5687), G200 superfine (lot 9730), Sepharoses, and Dextran T-2000 (lot 8122) were products of Pharmacia Fine Chemicals. Biogel P-300

and Biogel Agaroses were from BioRad, while Ultrogel AcA44 was from LKB Products. Glass beads were 200 μm diameter beads used in reflective paint by the Kansas Highway Department. They were washed with 6N HCl before use.

All of the agarose products were washed before use by resuspending 1 volume of gel in 5 volumes of deionized water, letting the gel settle, and decanting the supernatant. This was repeated six times followed by three similar washes with buffer (0.025 M Tris · Cl, pH 7.4).

Light-scattering determinations were done using a modified Brice-Phoenix light-scattering photometer. Narrow slits were placed in front of the light source and immediately before the sample so that a beam of light approximately 1.0 mm wide and 10 mm high illuminated a sample in a round 13 × 100 mm pyrex test tube. Light was collected from a slit of the same size on the receiver nose piece to provide a very narrow angular resolution (calculated 1.5° angle subtended). For instance, water, a solution of 60% sucrose, or pure glycerol showed a change in light intensity of more than 100-fold on going from a receiver position of 0° to 3° from straight forward.

3. Results

As a preliminary screening technique for possible matrices to use in gel scanning chromatography I have done "spectra" on a Cary 14 through the visible region. With a scattering material, if the apparent absorbance in the visible region is greater than 2A it will certainly be unsatisfactory in the ultraviolet region. Much of my work is with proteins having chromophores which absorb visible light so that visible-region performance per se is of considerable interest, and matrices unacceptable for work in the ultraviolet may be acceptable for work in the visible region.

Ackers [3] reported baseline spectra obtained using scanning chromatography for a variety of matrices of the Sephadex series. My results obtained using a Cary 14 agree reasonably well with these (table 1). Agarose, in the Sepharose series, showed much more scattering, in addition to appearing somewhat brown in transmitted light. The BioRad agaroses were much superior in their light-scattering properties, although they are of the same nominal compositions Pharmacia Sepharoses.

Difference spectra between some pairwise combinations of matrices were run to compare the relative scattering power of matrices as a function of wavelength. When Biogel P-300 was scanned with Agarose A50M as a reference, the apparent absorbancy was 0.36 at 700 nm, falling slowly to 0.29 at 375 nm and to 0.16 at 300 nm. The two matrices thus show rather different "turbidities" at a long wavelength but at first glance a similar wavelength dependence of scattering. Analysis of the scattering vs. wavelength indicates a rather different dependence, however. (See below.)

Other pairwise comparisons between matrices of identical nominal pore size but different particle size (e.g., regular versus superfine Sephadexes) indicated that the smaller particles had a greater light-scattering capacity at long wavelengths but about the same at short wavelengths. The effect of more extensive washing was tested for Agarose A15 M by packing gel into small columns (10 ml) and washing them with 500 ml of buffer. Scans taken on this extensively washed material showed that for A15 M there was a constant absorbancy difference (0.1) between the 50–100 mesh and the 100–200 mesh beads from 700 to 300 nm. These more extensively washed beads, from which the fines had not been decanted as described in section 2, showed greater light scattering at all wavelengths between 700 and 300 nm than gels washed by decanting of fines.

An additional measure of the acceptability of gel matrices for direct scanning chromatography is determination of the angular dependence of light scattering. If the light is scattered through relatively small angles, use of a wide collection angle will reduce the effective losses to reasonable values and the pathlength difference between directly transmitted and scattered light should be small.

Typical angular dependencies of light scattering are shown in fig. 1 for a number of matrices. The differences between spectra taken at 436 and 546 nm give an indication of the nature of the scattering function for various matrices. The ideal matrix would be one that scattered light equally at all wavelengths. Biogel P-300 is the best in this respect, presumably because it has a relatively narrow size distribution. However, in actual experiments of gel filtration capaci-

Table 1
Apparent absorption at selected wavelengths for several gel matrices

Gel type [a]	A_{700}	A_{500}	A_{300}
BioRad Agaroses			
A0.5M 100-200 mesh, 10% Agarose	1.48	1.53	1.86
A1.5M 100-200 mesh, 8% Agarose	1.29	1.35	1.71
A5M 100-200 mesh, 6% Agarose	0.99	1.05	1.60
A15M 100-200 mesh, 4% Agarose	0.54	0.65	1.41
A15M 100-200 mesh, 4% Agarose [b]	0.58	0.72	1.73
A50M 100-200 mesh, 2% Agarose	0.48	0.62	1.39
A5M 50-100 mesh	0.80	0.86	1.60
A15M 50-100 mesh	0.44	0.50	1.45
A15M 50-100 mesh †	0.48	0.62	1.63
A50M 50-100 mesh	0.52	0.61	1.31
Sephadex Sepharoses			
CL-6B	1.52	1.95	>2 (487 nm) [c]
6B 100	1.43	1.84	>2 (462 nm)
CL-4B 200	1.06	1.54	>2 (413 nm)
4B	1.14	1.51	>2 (399 nm)
Sephadex G200R	0.68	0.83	1.25
Sephadex G200-120	0.51	0.67	1.25
Sephadex G200	–	0.65	1.08
Sephadex G200S	1.19	1.38	>2 (325 nm)
Ultrogel	1.38	1.53	>2 (334 nm)
Biogel P-300	0.87	0.95	1.51

[a] Biogel agaroses had the following lot numbers: A0.5M 100-200 mesh 176752, A1.5M 100-200 mesh 176202, A5M 100-200 mesh 174322, A5M 50-100 148231, A15M 100-200 mesh 166762, A15M 50-100 mesh 166761, A50M 100-200 mesh 168882, A50M 50-100 130551.
Sephadex Sepharoses had the following numbers: CL6B, 2108; 6B-100, Sigma 38C-0125; CL4B200, Sigma 376-0023; 4B, 8217. Sephadex G200R was lot 5687 from Pharmacia; Sephadex G200R-120 was lot 156-0141 from Sigma; Sephadex G200S was lot 9730 from Pharmacia; Ultrogel was lot 9067 from LKB Products; Biogel P-300 (100-200 mesh) was lot 141562 from BioRad.
[b] Gel was prepared by washing a small column of 10 ml with 500 ml of buffer.
[c] Wavelength at which absorbancy reaches 2.0.

ty it has performed quite poorly compared to Sephadex (L. Davis and W. Vogt, unpublished observations), so that it may be optically acceptable but physically unacceptable.

The light-scattering intensities shown in the figure are not absolute since the pathlength being observed varies with the angle of observation for a cuvette of finite size. They do, however, represent the system as it would be observed by a photomultiplier tube placed close to the column with various angles of observation accessible. That is, they are relevant intensities for the gel scanner system. The striking feature of these patterns is the extreme concentration of scattered light in the forward direction. The figure is plotted on a logarithmic intensity scale so that if the maximum intensity is considered as "zero A", then the relative absorbance at 30° is nearly "1A" for Sephadex and "3A" for Agarose A50M. Thus much more than 90% of light is collected in less than ±30° from the forward direction.

To demonstrate that the scattering was a function of the presence of relatively few large scattering centers rather than a large number of independent small scattering centers, I did the following experiment. Equal concentrations of G200 regular and G200 superfine, G-75 regular, and Dextran T-2000 solutions (M.W. 2×10^6) at equivalent concentration of dextran (3.6%) were examined at different dilutions. The in-

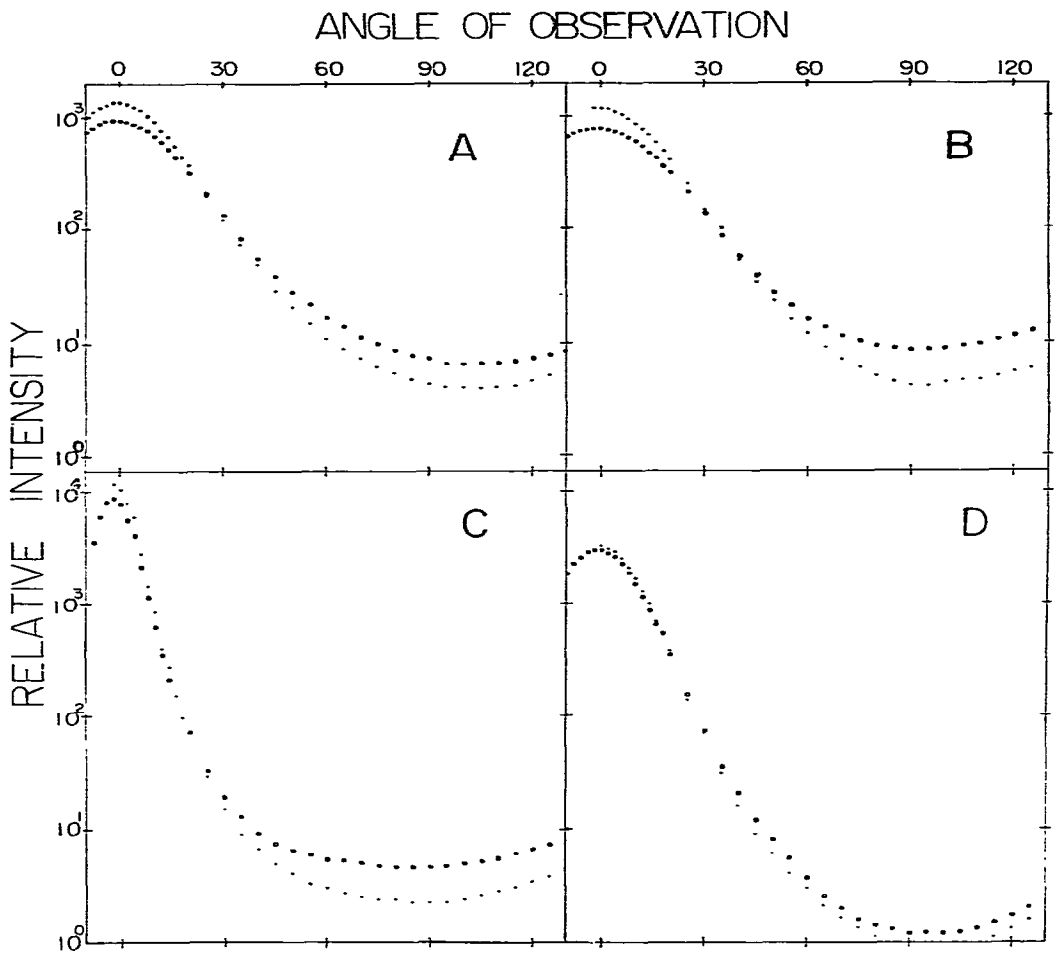

Fig. 1. Angular dependence of light scattering for several gel matrices at two different wavelengths of visible light. Light-scattering patterns were determined with the indicated gels packed by gravity into 13 × 100 mm pyrex culture tubes. The spectra were obtained as described in Methods, at 436 and 546 nm. Light intensities (ordinate) are plotted on a logarithmic scale against angle of observation (abscissa). Solid symbols, 546 nm; open symbols, 436 nm. Part A, Sephadex G200R in 0.025 mM Tris, pH 7.5; part B, Ultrogel in H_2O plus 0.02% NaN_3; part C, Agarose A50M (150-300 μm) in 1 mM Tris EDTA plus 0.02% NaN_3; part D, Biogel P-300 in 0.025 M Tris·Cl, pH 7.5.

tensity of scattering observed at a fixed angle of observation (90°) was proportional to concentration of the Sephadex beads. The scattering from G200 superfine was significantly greater than that from G200 regular for a given weight concentration (with the same extent of cross-linking). This would be expected since the particles of the superfine material are considerably smaller than the regular material and hence there are more beads per unit weight. Scattering by G-75R was very similar to that by G200R on a number basis, that is, half as great on a weight basis.

With dextran solutions above 0.03%, the scattering was not proportional to concentration. This is expected since the number of independent scattering centers in the observed volume (about 0.1 ml at 90°) is much larger for Dextran T-2000 than for the Sephadex dilutions – of the order of 10^{10} particles per 0.1 ml of a 3.6% solution of T-2000, compared to about 3000

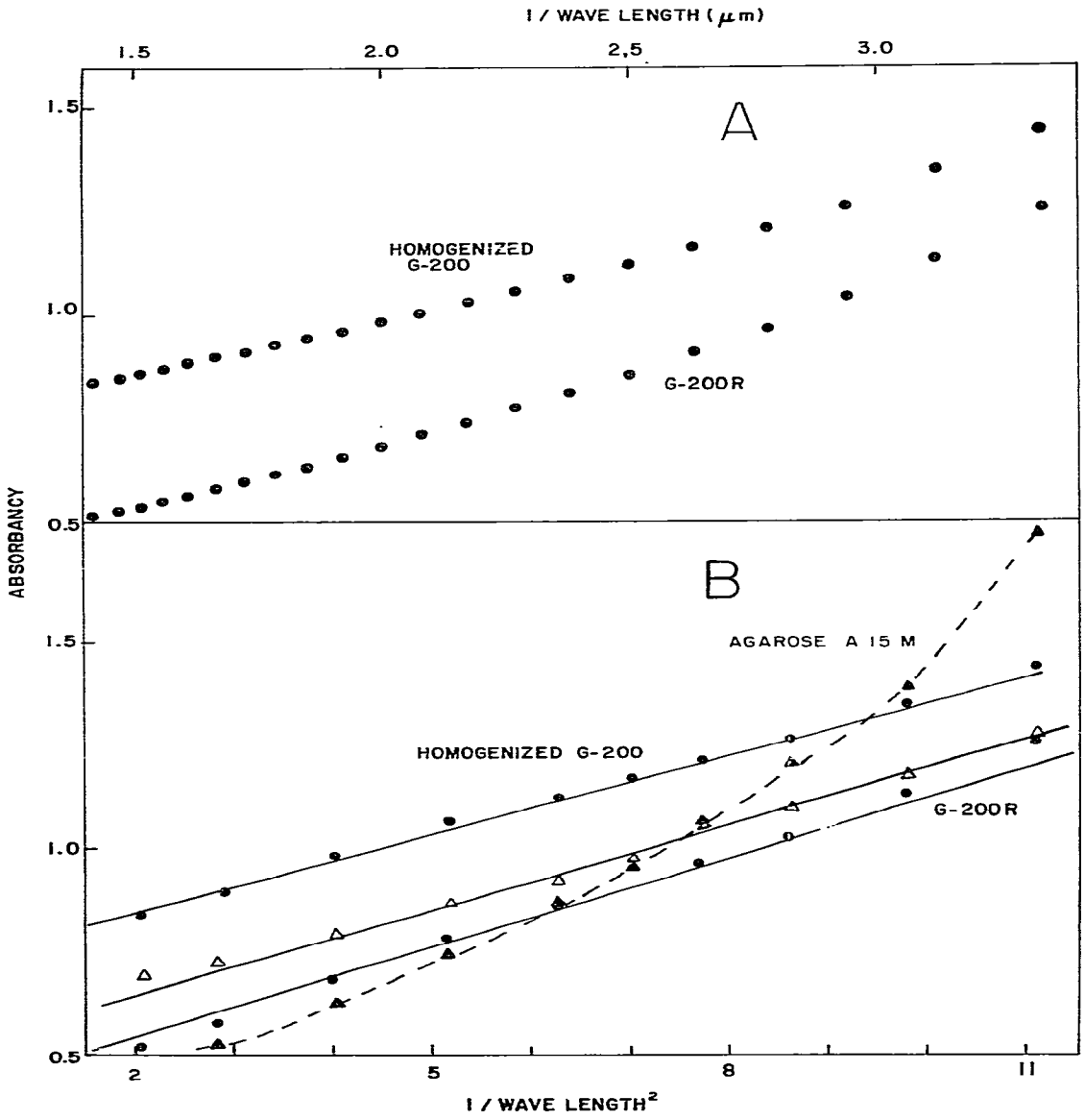

Fig. 2. Light scattering by matrices as a function of particle size and matrix type. (A) Light scattering by Sephadex G200R and homogenized G200R plotted as apparent absorption versus 1/wavelength. Solid circles are G200R and open circles the homogenized G200R. Gels were fully settled in 3 ml cuvettes. (B) Comparison of the apparent absorption of Sephadex and agarose. The Sephadex lines, solid and open circles, are the same data as in (A) but plotted as a function of 1/wavelength^2. The agarose used was A15M, washed in a small column as described in text. Solid triangles are plotted as apparent absorption versus 1/wavelength^2, while open triangles are the square root of the apparent absorption versus 1/wavelength^2.

particles for regular Sephadex packed solid in a column. At the greatest dilution examined, 1/30, there would be only about 100 Sephadex particles per 0.1 ml while there would be about 3×10^8 per 0.1 ml in the dextran solution. At such high concentrations there is a good chance of interference by one particle with the scattering pattern of another. The Sephadex bead may be viewed as an aggregation of smaller dextran particles into large domains wherein the interference by one domain or another is assured by their close proximity. This may be why the scattered intensity at 90° per unit weight is so much lower for Sephadex than for Dextran T-2000.

One needs to distinguish between the scattering intensity at 90° and the apparent absorbancy (turbidity, extinction) at 0° (forward). When one simply observes the loss of light attributable to scattering from the forward direction, a solution of Dextran T-2000 is found to be a much weaker source of scattering than Sephadex G200; it is nearly transparent in transmitted visible light. The scattering intensity of the dextran solution increases roughly as the inverse fourth power of wavelength; that is, it is Rayleigh-type scattering, while that for Sephadex increase inversely with a much lower power of wavelength. The following experiment indicates that scattering by Sephadex has contributions from both large and small scattering domains (figs. 2A and 2B) but primarily from large domains. A suspension of Sephadex G200 regular in 0.025 M Tris buffer was homogenized in a Potter-Elvehjem homogenizer. The homogenized material was allowed to settle for 3 days in a 3 ml cuvette, and the apparent absorption spectrum was determined. Serial dilutions of the suspension were also made, and absorption at selected wavelengths was recorded. There was about a two-fold increase in the apparent absorption as a result of breaking up the Sephadex particles. This was nearly constant at all wavelengths and relatively constant over a range of concentrations, except that the effect was less than expected when completely settled gel was used (table 2). Homogenization would be expected to break up the macroscale of the particles but not alter the microscale scattering centers. The observed results suggest that scattering at short wavelengths comes in part from small scattering domains while the scattering at long wavelengths comes predominantly from large domains whose contribution at short wavelengths is of less importance than that from small domains.

Table 2
Relative absorbancy of regular and homogenized G200 *

Dilution	Wavelength of observation		
	300	500	700
1/9	2.26	2.1	1.80
1/5	2.22	2.29	1.83
3/11	1.70	2.08	2.26
1/3	1.55	2.31	2.16
Undiluted	1.14	1.45	1.64

* G200 regular was used directly in 0.025 M Tris buffer, pH 7.4, or homogenized 3 min in a motor driven Potter-Elvehjem homogenizer and then observed. Settled gels were scanned (fig. 2) and then several dilutions were examined at selected wavelengths. Apparent absorption values are shown as the ratio of homogenized/regular.

In fig. 2A the apparent absorption for undiluted samples is plotted as a function of 1/wavelength, while in fig. 2B it is plotted as a function of 1/wavelength^2. It can be seen that for G200R the fit to an inverse square of the wavelength overcorrected the nonlinearity apparent in part A, at most wavelengths except near 300 nm. For the homogenized G200R the fit was similar. A fit to a power curve indicated that for homogenized G200 the inverse of 1.6 power of wavelength gave the best fit.

The results with Sephadex are strikingly different from those obtained with agaroses. In fig. 2B, the absorption of Agarose A15M is shown to be markedly curved. A plot of the square root of the absorption versus 1/wavelength squared gave a nearly straight line. That is, for agarose the scattering varied nearly as the inverse fourth power of wavelength; it was Rayleigh-type scattering. This same dependence on the inverse fourth power of wavelength was found to hold for Agarose A15M, down to 210 nm. For measurements in the ultraviolet region of the spectrum, 2 mm pathlength cuvettes were used.

The variation in pathlength caused by light scattering was examined by saturating gel matrices with a solution of hemoglobin, which would be relatively excluded from the gel beads and would absorb light in the appropriate region of the spectrum. If the pathlengths were identical for light scattered through different angles, the apparent absorbancy of the hemog-

lobin solution should remain a constant over different angles of observation. If the pathlength at different angles were a simple function of the shape of the sample tube, the apparent absorbancy of the hemoglobin solution should decrease as the angle of observation departed from zero (forward). The exact shape of the geometry-dependent light-scattering function for a given system depends on the areas illuminated and observed. For the present apparatus, the pathlength should decrease by a factor of about 2 between 0° and 90°. However, since the hemoglobin absorbs an appreciable fraction of the light, there would be less light available to be scattered forward in the long pathlength so that the geometric effect would be reduced as a function of the intensity of absorption. Determination of "absolute" partition coefficients

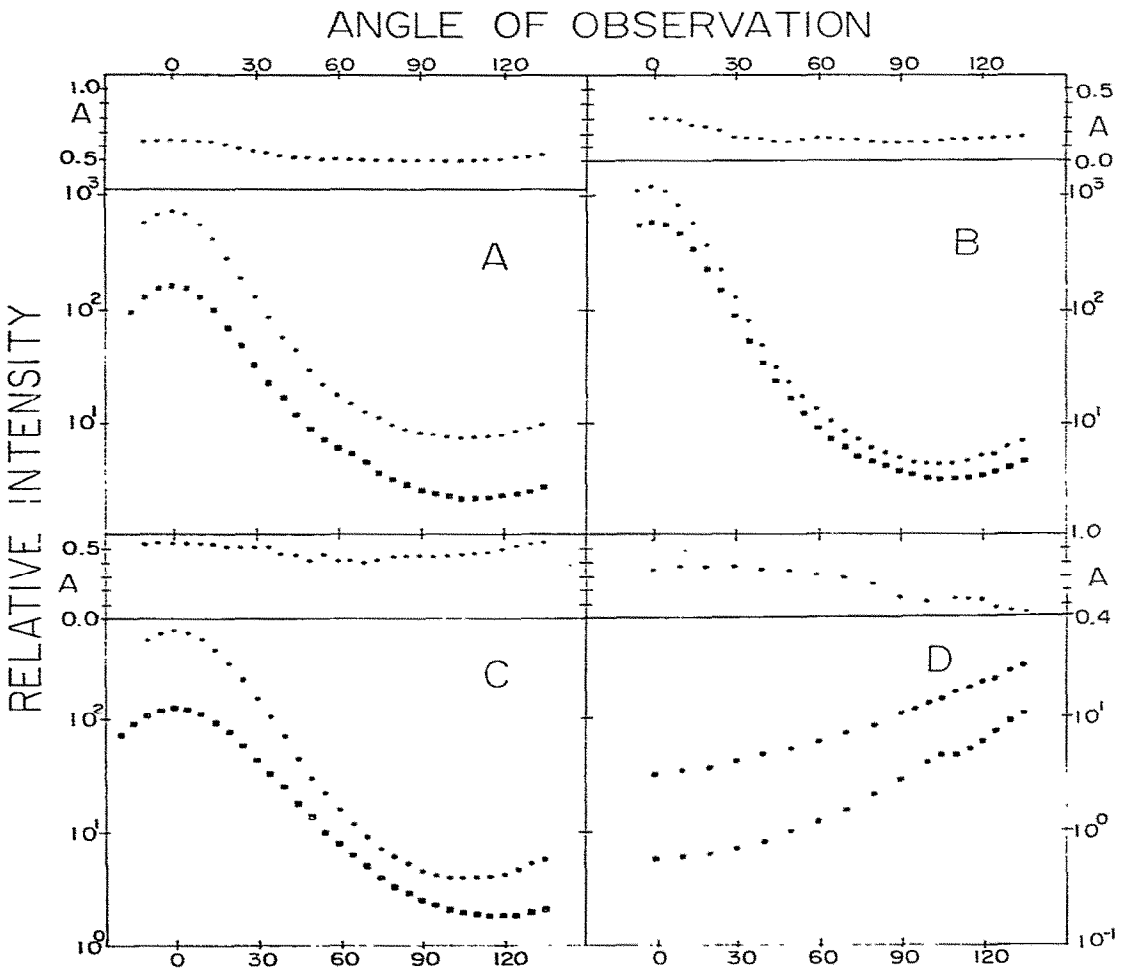

Fig. 3. Light scattering by Sephadex or glass beads and absorption by hemoglobin. Relative intensity of scattered light was determined as indicated in section 2 for matrices suspended in 0.025 M Tris or saturated with a solution of 1 mg/ml hemoglobin in the same buffer. Solid circles, gel alone; solid squares, with added hemoglobin. In each part of the figure the fraction of light absorbed by the hemoglobin solution is shown above the scattering patterns as a function of wavelength. (A) Sephadex G200 superfine at 436 nm; (B) Sephadex G200 superfine at 546 nm; (C) Sephadex G100 superfine at 436 nm; (D) glass beads at 436 nm. The apparent absorbancy of the hemoglobin solution at 436 nm in the absence of matrices was ~0.95, while at 546 nm it was ~0.35.

depends on the assumption of uniform pathlength for light directly transmitted and light scattered through the angle of collection used in a particular scanner configuration. If the pathlength is not constant, because of either geometry or scattering, the derived partition coefficients are not exact, although they may form a self-consistent series as long as the scanner configuration, angle of collection, and extent of scattering by different matrices are similar.

In fig. 3 some results for angular dependence of light scattering and absorption are presented. For each part of the figure the scattering patterns as well as the apparent absorbancy of the hemoglobin solution are shown. In another experiment (not shown) the partition coefficient (concentration inside versus outside gel) for hemoglobin in G200 superfine was determined to be ≈0.65 by difference spectroscopy in the Cary 14 over a range of wavelengths. This partition coefficient was in reasonable agreement with the apparent absorbancy of hemoglobin plus G200 versus hemoglobin in solution (0.65/0.95) obtained in the light-scattering apparatus which gives a partition coefficient of 0.68 at 0° (fig. 3). The agreement at 546 nm was less satisfactory. An absorbancy of 0.3A with matrix versus 0.35A without matrix in the light-scattering apparatus was observed, which gives a partition coefficient of 0.86. The apparent partition coefficient from the light-scattering curves with G100 superfine was 0.55 at 436 nm. The difference of partitioning between matrices is in the right direction since hemoglobin is nearly excluded from G100, whereas it is partially included in G200. The apparent absorbancy in the presence of glass beads was much higher than it should have been, suggesting that the small amount of light transmitted in the forward direction by this matrix had undergone multiple scattering events, and hence had an apparently longer pathlength. The glass beads being used had an excluded volume fraction of 0.36, while the apparent hemoglobin absorbancy in the presence of beads was 0.75A, which is more than twice the physically possible absorbancy and implies a partition coefficient of 0.79, which is also physically impossible.

The angular dependence of the observed partition coefficient is of particular importance for operation of direct gel scanning systems, since the effective comparison of scanner results with partition coefficients determined by other means depends on a reasonable constancy of the observed values. In fig. 3, I have plotted the apparent absorbancy of the hemoglobin solutions as a function of angle of observation. It may be observed that there is a decrease in the apparent absorbancy at large angles of light collection. The relative decrease is less at 436 nm than at 546 nm in G200 superfine. The decreasing absorbancy with increasing angle of observation is a function of the geometry of the system. However, if the light observed at wide angles had undergone multiple reflections such that it had an increased pathlength through the gel matrix, the apparent absorbancy would be increased. These two effects (geometry and scattering) would be in opposite directions and thus might be partially cancelling. It is difficult to make a quantitative analysis of the expected versus observed absorbancies at all angles to determine the relative contributions of each effect. The most important conclusion to be drawn is the simple empirical observation that since much more than 90% of all light is scattered to less than 30°, the error resulting from scattering at wider angles is relatively unimportant to conventional scanner operation. However, there must be some small pathlength error even at smaller angles which will affect calculated partition coefficients based on absorbancy measurements. The extent of the error will vary with exact scanner configuration.

For Sephadex beads, which are compressible, measurement of the excluded volume is difficult and small pathlength effects due to scattering are hard to exclude rigorously. The partition coefficient for a completely excluded molecule such as blue dextran ought, however, to be identical for direct scanning and for a volume displacement technique such as that described by Ackers [4] if there are no pathlength errors. This comparison has not yet been reported so far as I am aware. The most persuasive argument that there are no appreciable pathlength errors introduced in the scanning methods by scattering effects is the observation of Brumbaugh and Ackers [1] that the absorbancy of a solution of myoglobin above and within a matrix showed a linear correlation over a wide range of wavelengths, from 220 to 440 nm. Of course, if the pathlength error due to scattering increased simply as the inverse of a low power of wavelength on going from 440 to 220 nm, which is the case for large particle scattering [4], the effect would be a small nearly linear increase in pathlength in the presence of the gel.

This would produce a systematic nearly linear deviation of apparent partition coefficient as a function of wavelength, the magnitude of which would depend on scanner configuration and which might be quite small.

4. Discussion

In order to apply the method of van de Hulst [4] and treat the light scattering as anomalous diffraction we need to show that gel matrix particles meet two criteria. First, the refractive index increment relative to solvent must be small, and, second, the beads must behave as large independent scattering centers. The refractive index increment for a dextran gel such as Sephadex may be estimated from the water regain and the value for the refractive index increment of dextran solution, assuming that the cross-linking agent has relatively little effect on the refractive index [6].

The size of the particles can be determined in a hemacytometer, or may be accepted as stated by the supplier. The average size for regular G200 is near 80 μm (range 40–120 μm). Inspection of hydrated beads in a hemacytometer indicated that this was a reasonable estimate. As shown in fig. 2, the beads do behave as independent scattering centers to the extent that scattering is proportional to number of beads per pathlength. Thus the method of van de Hulst [4] may be applicable.

To calculate the expected intensity of scattering we can use the following equation:

$$i = 4\mu^2 x^2/(4\mu^2 + \theta^2)^2,$$

where μ = refractive index increment, $x = \pi d/\lambda$, d is the diameter of the bead, θ = angle of observation, and i is the apparent intensity of the scattered light. To simplify calculations we assume that the packing factor for a gel column is 0.5, i.e., that the gel beads take up half the space in the column. Then the refractive index increment in a gel bead is two times the apparent dextran concentration of the beads determined from water regain, times the refractive index increment for a dextran solution of the same overall concentration. Typical G200 beads have a water regain of 20 g/g dry weight. A 5% dextran solution has a refractive index increment, relative to water, of 0.008 [7] so that 0.016 is a reasonable value for μ. With a light source of 0.5 μm wavelength the relative intensity at 0° is 250 600. At 10° the relative intensity is 2.6 while at 90° it is 3.2×10^{-3}. Essentially all of the light is collected between 0° and 10° in this case.

Van de Hulst [4] has supplied an "altitude chart" showing relative intensity of scattered light at different angles of observation for large particles of low refractive index. Results obtained by reading off the chart are in agreement with those calculated as described above. In either case there is a simplifying assumption that the particle is a uniform sphere of low refractive index increment and that the scattering by one particle is independent of that of its neighbors. When the beads are in contact, this model obviously cannot hold and the total scattering observed must be the sum of scattering from a large number of beads of different sizes with different angular dependencies, with some amount of mutual interference of scattering effects. Thus the overall qualitative conclusion is clear, but the experimentally observed scattering is somewhat more complex than a simple summation of the scattering per particle times the estimated number of particles.

The particles may be more accurately considered as containing a series of randomly distributed domains of greater and lesser dextran density, rather than as uniform spheres [6]. As long as the domains responsible for scattering are large relative to the wavelength of observation, the consequences are similar. That the beads are more like a sponge than a uniform gel is easy to demonstrate. A uniform gel, when immersed in a solvent of appropriate refractive index, will show no scattering and hence will seem invisible. A sponge with the same average refractive index but wide density fluctuations over different domains will remain visible at any solvent refractive index. Suspending Sephadex or polyacrylamide beads in glycerol or sucrose solutions (0–70% tested) reduces but does not abolish their visibility, whereas glass beads can easily be rendered invisible (non-scattering) in appropriate organic solvents.

Both nonuniformity in bead size and the variable density within a given bead must contribute to the relatively broad angular scattering observed. One can compare the relative intensity of scattering shown in fig. 2 for G200R and G200R (homogenized) to see the effect of bead size. Although light scattering does

not result solely from the beads as discrete entities, the beads must behave to some extent as coherent centers of scattering, or the scattering would not vary with number of beads per unit volume, but rather with the bead porosity. Scattering from G-75R is approximately the same as from G200R on a volume basis (number of particles) while it is one-half as great as a dextran concentration basis.

The results in table 2 are shown for "0°" only, where intensity of observed light will not depend solely on particle number. The relative intensity of light observed at 0° varies with both wavelength and concentration of beads in different nonlinear ways for different types of gels. Interpretation of these patterns is too complex to attempt here.

The light-scattering properties of glass beads are easier to interpret. In this case the term μ (refractive index increment relative to water) is large – of the order of 0.2. If the size of the particles is uniformly 200 μm, and they are all smooth spheres, they can be treated similarly to drops of water in air ($\mu = 0.33$), for which a large body of data is available [4]. For the case of a large water droplet about 5% of the light will be immediately reflected at the surface and 5% will undergo multiple reflections. The remaining 90% will undergo refraction through angles 0–90°. About 55% of the total light appears between 0° and 30°, 32% between 30° and 60°, 3% between 60° and 90°, and the remainder at greater angles. Thus more than 40% of the light incident on each particle may undergo further scattering before reaching the receiver. A large fraction of light received at any angle must have undergone multiple scattering events since there are several thousand beads in the observed volume.

In searching for a matrix, one would like one with a scattering cone strongly focused in the forward direction and very little in the backward direction if there is to be no distortion of absorption bands [2]. This may eliminate from consideration as matrices controlled-pore glass beads coated with dextran, which could be useful for studies of aggregation of high molecular weight materials. At least it is clear that data could not be treated by simple methods of absorption spectroscopy. However, the importance of being able to examine interacting systems of higher molecular weight may in time justify extension of the technique of direct scanning to such nonideal matrices.

The angular dependence of the light leaving a column has some practical implications for scanner design as discussed by others [8,9], suggesting that we should collect a relatively wide angle of ±20° from the forward direction. The relatively weak backscattering may explain why there is no deviation from Beer's law in the presence of absorbing materials. The average pathlength is altered only very slightly from the assumed pathlength since only light scattered forward is collected by the photomultiplier and essentially no light undergoes two or more backscattering events, which would lead to a significant increase in the deviation from the average pathlength.

Any matrix acceptable for use with presently designed scanners must have a relatively low refractive index increment and relatively large effective particle size. A few possible matrices have been tested for their light-scattering behavior, measuring apparent absorption, or the angular dependence of scattering, particularly large pore matrices. When choosing between matrices with similar and acceptable light-scattering losses, other factors, such as the resolving power in gel filtration, become more important than the light-scattering properties. New matrices with high resolving power for high molecular weight materials, and acceptable light-scattering patterns, are still being sought.

Among those currently available, Sephadexes appear to be clearly superior for work in the ultraviolet region. In the visible region of the spectrum, the agaroses have a definite advantage in resolving power for high molecular weight aggregates and an acceptable light-scattering baseline. However, in the ultraviolet they are unsatisfactory unless very short pathlengths are used or a scanner of exceedingly high photometric accuracy is devised. Porous glass would appear to be unsatisfactory in both regions of the spectrum both because of light losses and because of pathlength errors introduced by relatively large backscattering.

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