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TRANSMISSION-ELECTRON MICROSCOPIC OBSERVATIONS OF FREEZE-ETCHED POLYACRYLAMIDE GELS*

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SUMMARY

Structures of polyacrylamide gels were examined by transmission-electron microscopy of freeze-etched specimens. This experimental approach allows higher resolution than that achieved in previous studies and reveals structural details that correlate with empirical findings concerning the molecular sieving effect of polyacrylamide gels. The influences of the total acrylamide concentration, the cross-linking ratio, the different cross-linkers and the catalyst concentration were investigated. A comparison of polyacrylamide gel with agar gel revealed a structural difference in the two materials.

INTRODUCTION

Polyacrylamide gel was first employed as a support for electrophoresis of macroions, such as proteins, in 1959[1,2]. Due to the molecular sieving effect and the inert, apparently uncharged matrix that does not cause electroendoosmosis, polyacrylamide gel electrophoresis quickly became a favorite tool in analytical biochemistry. Many variations of the gel, as well as the electrolyte composition employed for electrophoresis, have added versatility to its use. For example, the introduction of the ionic detergent SDS to gel electrophoresis (for review see ref. 3) and the development of gel electrofocusing (for review see ref. 4) can be cited. In addition, polyacrylamide gels have entered the domain of modified dextrans and are frequently

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used for gel filtration, gel chromatography, entrapment of enzymes and, in modified form as a matrix for covalent attachment of protein[5,6]. Numerous publications describe directly the development of gel electrophoretic techniques; and if those investigations that make use of these techniques were included in a bibliography, the total number of references would certainly exceed 10,000. For instance, by the end of 1974 there were more than 1000 publications that dealt with gel electrofocusing, a branch of gel electrophoretic techniques introduced in 1968[4].

In spite of the apparent importance of this technique, investigations of the sieving matrix of polyacrylamide gels are rare and have been published only recently. Some investigators attempted to visualize gel structure with transmission-electron microscopy (TEM). However, these attempts failed and remained unpublished because dehydration and embedding of gels produced shrunken masses that lacked recognizable structures. Similarly, lack of structure has also been observed in gels prepared by the critical point-drying method and observed by scanning-electron microscopy (SEM)[7]. In two recent papers involving freeze-drying and critical point-drying under conditions that prevented the gel matrix from shrinking during dehydration, SEM revealed a spongelike closed cell structure[8,9]. The probability and possible development of these structures in polyacrylamide gels have been previously discussed[10].

MATERIALS

Acrylamide, bisacrylamide and N,N,N′,N′-tetramethylethylenediamine(Temed) were products of Eastman (Rochester, N.Y., U.S.A.); ammonium persulfate was purchased from Fisher Scientific (Pittsburgh, Pa., U.S.A.) and diallyltartardiamide (DATD) was from Polyscience (Warrington, Pa., U.S.A.). Bisacrylylcystamine was kindly provided by Dr. J. N. Hansen (College Park, Md., U.S.A.).

METHODS

Polyacrylamide gels were polymerized from commercially available constituents. Bisacrylamide was recrystallized from acetone[11]. The acrylamide stock solutions contained 50% (w/v) total acrylamide (T*) and appropriate amounts of the crosslinker (C*), which was either bisacrylamide, DATD or acrylylcystamine. Crosslinkers other than bisacrylamide were exchanged on a mole-to-mole basis. For polymerization, stock solutions of 1.2% (v/v) Temed and of 0.25% (w/v) ammonium persulfate were used; all stock solutions were stable for several weeks when refrigerated.

Gel mixtures consisted of one part Temed stock solution, one part persulfate stock solution and the appropriate volume of acrylamide stock solution. The mixture was diluted with water to a final volume of 10 parts. This composition provided a lag period of 10 min at room temperature before the on-set of polymerization. In order to allow ample time for complete polymerization, the gels were held in a chamber for at least 2 h after they had been cast. The gel chamber consisted of two pieces of microscopical slide glass; the pieces were separated by two spacers (mi-

* T is the concentration of total acrylamide (acrylamide + cross-linker) in % (w/v); C is the cross-linkage, defined as percent of T, which is cross-linker.

croscope cover slips of No. 2 thickness from Fisher Scientific). As a result, gel slabs of 0.2 mm thickness were cast. These thin gels permitted easy identification of the fractured faces and the unfractured glass-adjacent surfaces. The gel chamber, which was held together by a clothespin, was cleaned with water and acetone before use.

Freeze-etching of the gels was accomplished in a modified Denton DFE-2 freeze-etch module operated on a Denton DV-503 vacuum evaporator. A resistance monitor equipped with a power cut-off device[12] was used for evaporation of some of the platinum shadow films.

Specimens consisted of small pieces (approximately 1 × 3 mm) cut from the 0.2 mm thick gel sheets. These thin slices were placed in complementary specimen holders[13], frozen individually by rapid immersion into partially melted but re-freezing Freon 22 and mounted in the special complementary specimen cap that was subsequently mounted on the pre-cooled stage (—150°) of the dry nitrogen gas-filled freeze-etch module. A vacuum of $5 \cdot 10^6$ Torr was obtained, and specimens at 150° were fractured by rotation of the cold (—196°) shroud. All specimens were warmed to —98° and etched (vacuum sublimation of the ice surface) for 10 min. Then specimen temperature was lowered to —150°, and platinum shadow and carbon replica films were deposited by vacuum evaporation onto the freeze-etched surface.

Finally, specimens were removed from the vacuum system and melted individually under the surface of distilled water. Polyacrylamide gel was removed from the replicas with Clorox (5.25% sodium hypochlorite) containing 10% NaOH. The cleaned replicas, consisting of only platinum and carbon were mounted on thin Formvar films for electron microscopy. Stereo-electron micrographs of all specimens were obtained in a JEOL JEM 100-B transmission-electron microscope equipped with a 60° top entry goniometer stage. Specimen tilt between the two electron micrographs of each stereo pair was a constant 10°. For simplicity, complementary replicas were prepared of all specimens, however, only a few were examined for complementary fit.

RESULTS AND DISCUSSION

To achieve the resolution that is necessary to visualize the ultrastructure of the polyacrylamide gels, replicas were prepared by the freeze-etch technique and were examined by TEM. This procedure involved the rapid freezing of small pieces of gel that were then fractured in vacuo. Throughout the ice sublimation procedure and the deposition of shadow and replica films, a temperature of —95° or colder was maintained to prevent collapse of the gel matrix. For assurance that collapse did not occur, complementary replicas (replicas of the two new faces of the specimen resulting from the fracturing procedure) were examined for complementary fit. Stereo-electron micrographs of these and all other replicas provide assessments of depth within the specimens and enable more accurate observations, the complementary fit and the retention of good 3-dimensional structure of the gel (Fig. 1) indicate that collapse of the gel during freeze-etching was not serious.

All polyacrylamide gels visualized so far by electron microscopy revealed a cellular structure in the submicron range. This is in contrast to the generally held assumption of a random meshwork of crosslinked individual polyacrylamide strands. The presence of comparatively large structures in the gel must not be an artifact

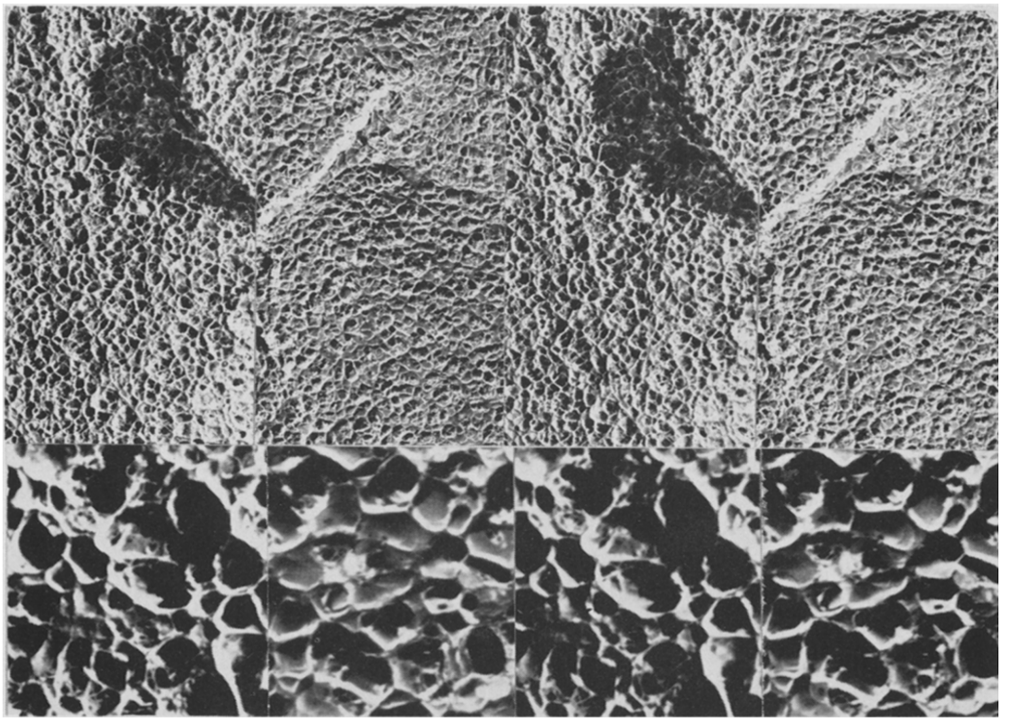

Fig. 1. TEM stereo images of complementary polyacrylamide gel surfaces (10% T, 6% C) after freeze-etching. The bars equal 1000 nm (top) and 400 nm (bottom). The technique employed for this investigation allows for the examination of both surfaces derived from a fracture of the frozen specimen. The fit of the complementary structures indicates that no changes of the structure occur in the further process of freeze-etching and coating.

due to freezing or dehydration, this is indicated by the fact that gels which are highly crosslinked with bisacrylamide turn opaque even in the hydrated state. Thus large structures are formed upon polymerization which interfere with the wavelength of light.

According to the observations of Flory[14], polymerization of synthetic chain molecules first produces high molecular aggregates that are in the sol state and therefore are not interconnected. This sol state exists in polyacrylamide, because homogeneous acrylamide mixtures that were polymerized in the gravity field of a centrifuge at *ca.* 20,000 *g* were rendered as continuous gradient gels. This indicates the occurrence of very large aggregates that sediment before they are crosslinked to the gel matrix[15]. The transfer of such aggregates from the sol state to the gel state starts abruptly at the gel point when they become crosslinked. This gelation causes a rapid reduction in the concentration of available sol particles[14]. As a result, when polymerization of gel occurs, the monomer is concentrated in some areas and necessarily depleted in others. A phase separation results that can be compared to the crystallization process or to the freezing-out of proteins into the interstices between ice crystals[16]. This process probably accounts for the appearance of the spongelike cellular structures visualized by SEM as well as by TEM.

Acrylamide is polymerized into long macromolecular strands that are inter-

connected by crosslinks. Therefore, a longitudinal array in bundles, which appear at high gel concentrations as membranes, must be assumed[14]. As the relative number of cross-links in the gel increases, the length of the originating polyacrylamide chains decreases; the distortion of the resulting membranes has been predicted to lead finally to clustering[17]. The formation of clusters has been demonstrated in highly cross-linked gels[9].

The possible configurations of the individual polyacrylamide fibers of the gel membrane are restricted by the effect of the "excluded volume"[18]. This effect results from the impossibility of selfintersections in the spatial arrangement of a macromolecular chain, causing polymerizing macromolecules to swell, and is the reason for the high pressure produced by gels of high concentration when cast in confining vessels like capillary tubes[19]. The degree of hydration-related swelling, which is determined by the ionic strength of the medium, implicates a competition between the gel and the free ions for the available water. In gels with low cross-linkage, polyacrylamide hydration and the related swelling never reach the saturation point[20]. Only in highly cross-linked gels does swelling become negligible. Thus the expansion of the polymerizing gel is balanced by the spatial restrictions of the cross-linked fiber network. This may result in a fine structure of the gel membrane that differs from a random array but resembles a lattice[9]. In the presence of attached water molecules, the macroion passage through such a lattice may be restricted. This could account for the molecular sieving effect of these gels.

A comparison of the cellular surface structure of gel observed by SEM[9] with those structures visualized by the method described in this study reveals a significant size difference. This difference may be explained as follows. In the SEM study, primarily surfaces of gels polymerized in glass tubes were examined. These surfaces displayed larger structural units similar to those illustrated in Fig. 2. Also, the development of pressure during gel polymerization in a confining vessel, such as a glass tube, causes a typical array of structures and favors larger structures[9]. To prevent these artifacts and to facilitate removal of acrylamide gel from the replicas, gels for the present investigation were polymerized in thin slabs under conditions in which excessive pressure could not develop. Furthermore, this procedure probably prevented the formation of the large cellular units except for those portions opposed to the glass surface (Fig. 2).

Comparison of the sieving properties of gradient gels, which were polymerized in capillary tubes (and were thus exposed to high pressure) or as slabs in a chamber that yielded to the expansion of the gel, did not result in significant differences. Thus, the substructure of gel membranes was apparently not grossly affected by structural conversions related to pressure.

In this paper, all gels examined by TEM were polymerized and prepared from deionized water. Therefore, the image of a low cross-linked gel does not represent the structure of such gel when kept in buffers of high ionic strength. With respect to sieving properties of such gels, the effective value of T in a 1% crosslinked gel approximates only one-half of the nominal value of T (ref. 21).

TEM images of the freeze-etched specimens were obtained from the interior of the gels that were exposed by fracturing of the frozen samples. A cross (Fig. 3) effectively illustrates the change in gel structure that results from alterations in the T/C ratios. The size of the structural units (both cell and pore sizes) is inversely

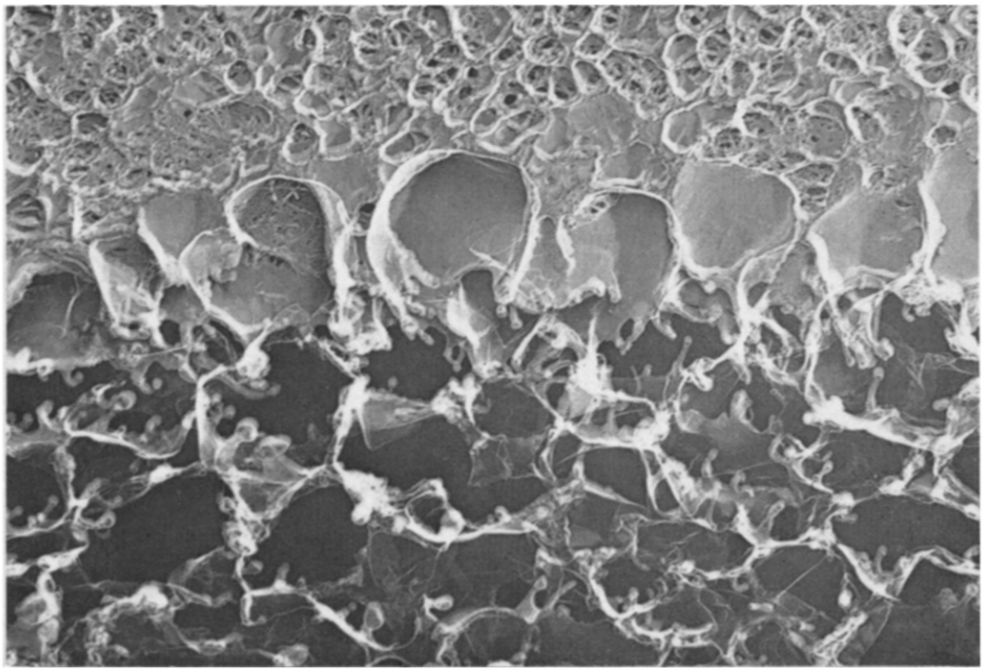

Fig. 2. TEM image of a 10% T, 1% C polyacrylamide gel; the bar equals 100 nm. The edge between the gel surface that was adjacent to the glass surface of the gel chamber (lower portion) and the fractured gel surface (upper portion) is displayed. The two surfaces show a significant structural difference. The larger structures formed at the glass interface have been illustrated in a recent scanning electron microscopical investigation[9]. The delicate fibrous structures of the fractured surface extend into the gross structure of the lower portion. Note that small pores in the large membrane-like structures at the glass interface are of the same dimension as those in the cross-fractured gel.

proportional to the acrylamide-comonomer concentration (T) and, therefore, directly related to the sieving properties of the gel (Fig. 3, horizontal series). The percentage of crosslinking (C) is reflected in the structures by a nearly parabolical function with structural units at a minimum size of about 5% C (Fig. 3, vertical series). This result is the morphological confirmation of the empirical fact that gels of the 5–7% C range produce maximum retardation for migrating macroions[17,22–25]. Thus, both low and highly cross-linked gels can be used as large-pore gels, as spacer gels in disc electrophoresis and for electrofocusing[26–29]. Migration of macromolecular particles through a large-pore gel matrix may occur through the possibly continuous passageways seen in Figs. 3 and 4.

Investigation of the retardation coefficient of proteins up to a molecular weight of $3.2 \cdot 10^6$ in gels of high cross-linkage[24] suggested that these gels could provide pore sizes suitable for particles of the subcellular level. Nevertheless, experiments (a) with gels of constant T (*i.e.* 5%) and a continuous concentration gradient of bisacrylamide (ranging from 0 at the bottom to 50% C at the gel top), (b) with gel gradients having approximately 1% T at the top at a constant cross-linkage throughout the gel and (c) with gels polymerized out of bisacrylamide alone [*i.e.* 2% (w/v)] indicated that the pore size of polyacrylamide has an upper practical limit. Thus,

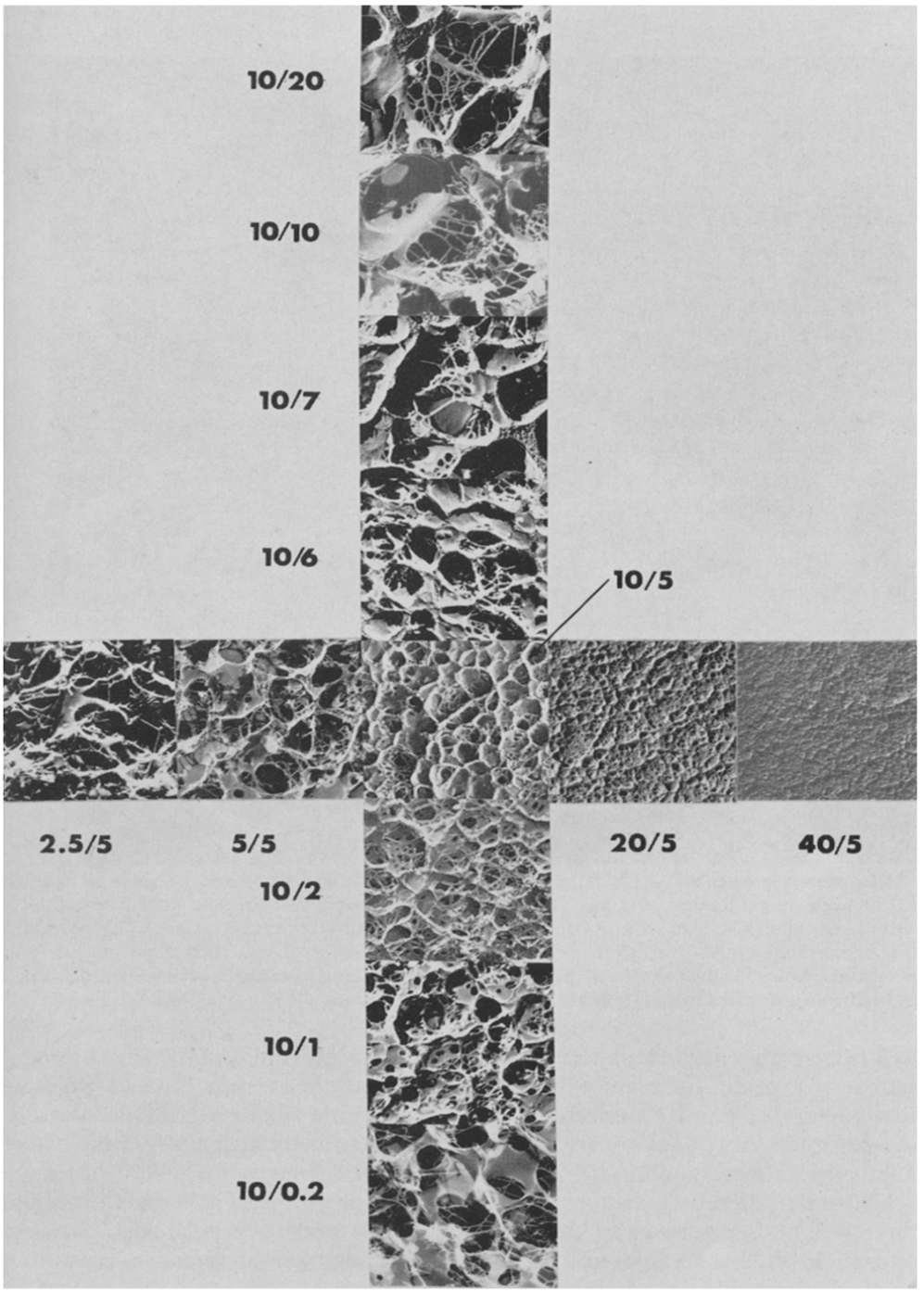

Fig. 3. TEM images of polyacrylamide gels with varying concentrations of total acrylamide (T) and varying concentrations of the cross-linker bisacrylamide (C). The values shown are T/C; the bar equals 400 nm. In the horizontal series, C remains constant and T increases left to right. This series reveals a decreasing cell as well as pore size, with increasing T, a relationship of gel structure proportional to the known sieving properties of the gels. In the vertical series, T remained constant and C increased from bottom to top. This series reveals a nearly parabolical function of the gel structure with a minimum size of *ca.* 5% C and confirms previous findings concerning the relationship between crosslinkage and sieving in the gel.

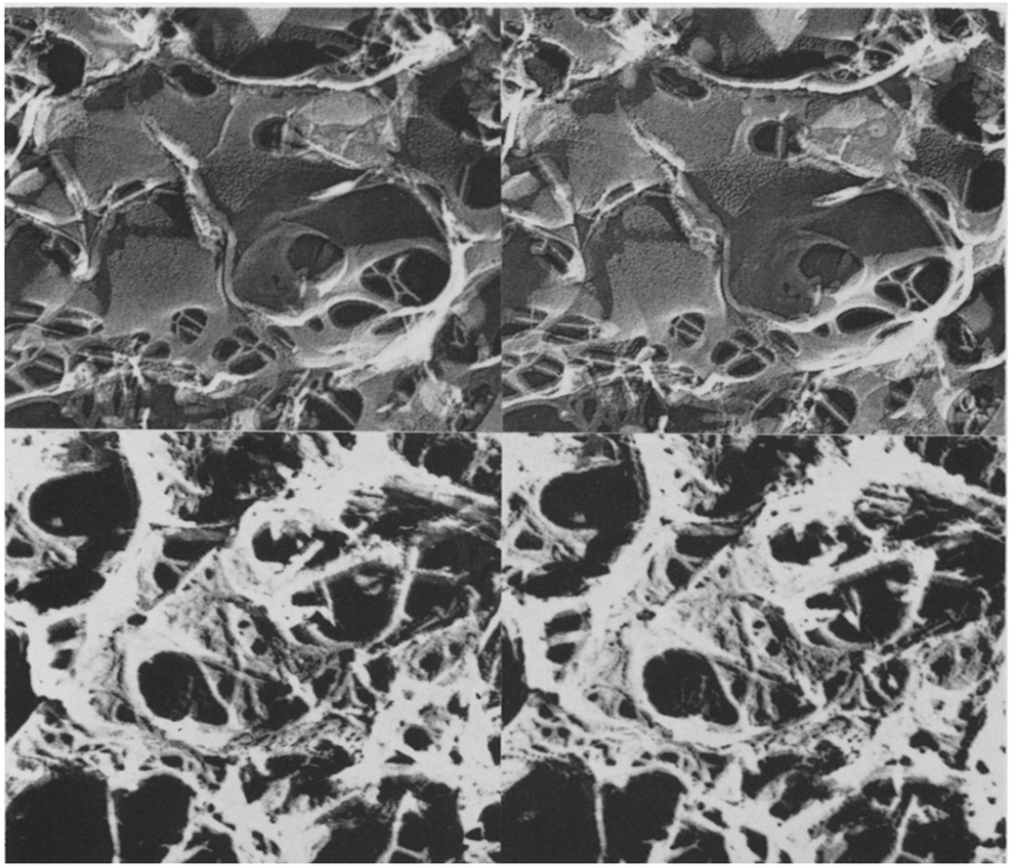

Fig. 4. TEM stereo images of a 5% T, 5% C polyacrylamide gel (top) and of an 8% agar gel (bottom) at high magnification; the bar equals 100 nm. While polyacrylamide becomes organized into membranes, agar gels stay as a fibrous meshwork even at high concentrations. The polyacrylamide gel membranes do not display a visible structure, but they resolve at their edges into delicate networks leaving holes for the passage of particles. The translucent appearance of the edges of vertical surfaces is due to the carbon replica film.

tomato bushy stunt virus (a spherical particle of $9.3 \cdot 10^6$ molecular weight and 30 nm diameter[30]) could be readily separated from southern bean mosaic virus (a spherical particle of $6.6 \cdot 10^6$ molecular weight and 25 nm diameter[31]). Alternatively, tipula iridescent virus [a spherical particle of *ca.* 130 nm diameter and a molecular weight of approximately $1000 \cdot 10^6$ (ref. 32)j and latex spheres that were tested up to 0.5 μm barely entered large-pore gels. Alignment phenomena allow multimillion-molecular weight DNA to enter deep into such gels and tobacco mosaic virus (a rodlike particle of 300 $\times$ 15 nm and *ca.* $40 \cdot 10^6$ molecular weight[32]) reaches a position close to that of the spherical bushy stunt virus[15].

Gels having intermediate and high values of T and a cross-linkage close to 5% C do not provide large passageways for unhindered migration, they are distinguished by the prevalence of rather homogeneous membranes (Fig. 3). A possible substructure became apparent upon fracturing of these gel membranes[9], however,

examination of the membrane surface of freeze-etched specimens at high magnification does not reveal a porous organization smaller than the scattered, clearly observed pores (Fig. 4).

The membranous types of gel are used for instance as separation gels in disc electrophoresis; the migration of a particle through such a gel includes its passage through spaces of the larger cells that could be described in terms of free electrophoresis and passage through the membranes where molecular sieving and thus separation according to size and shape may occur. Therefore, after electrophoresis, it is consistent to find protein primarily attached to the gel membranes[9].

Agar gel is used to separate large particles such as viruses[33], it must provide larger pores than polyacrylamide gels of comparable concentration. The agar gels are distinguished by the lack of membrane formation that must provide the major obstacle for migrating macroions in polyacrylamide gels of comparable concentration[34] (Fig. 4).

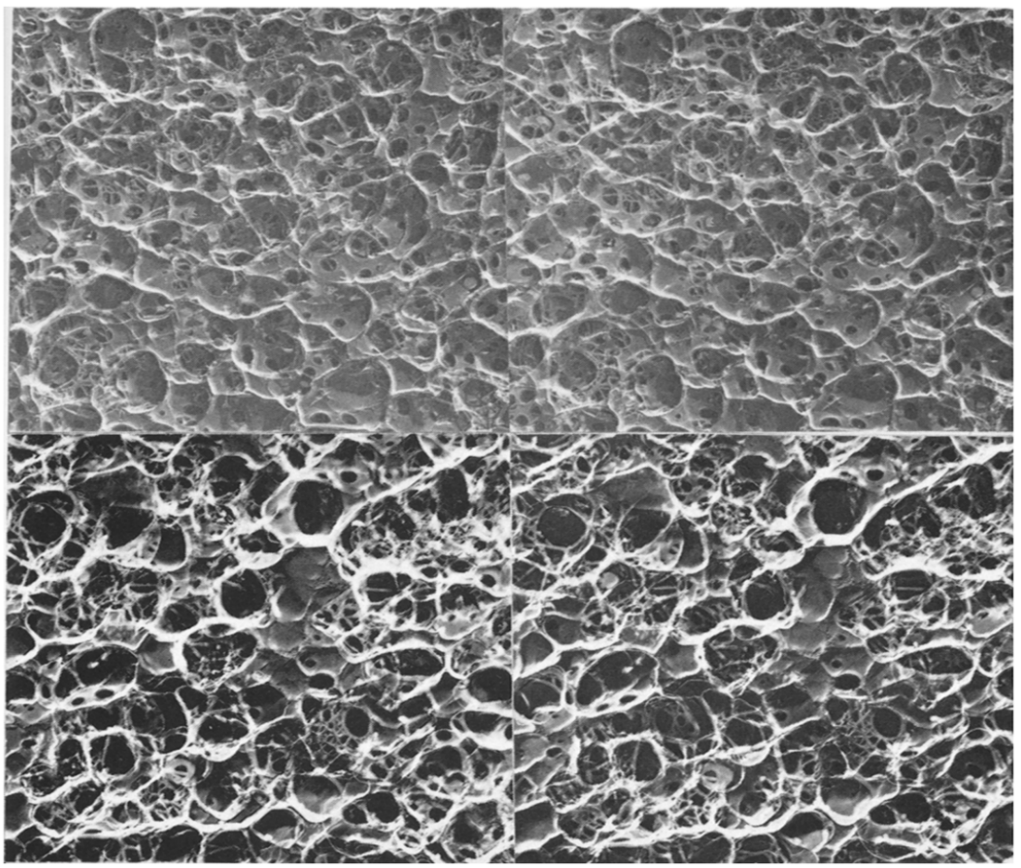

Fig. 5. TEM stereo images showing polyacrylamide gels that are cross-linked by diallyltartardiamide; the bar equals 400 nm. Top: gel of 5% T and 5% C. Bottom: gel of 10% T and 20% C. In spite of the differences in both values of T and C, little difference can be seen in the electron-microscopical structure. This contrasts sharply with the structural differences observed in gels cross-linked with bisacrylamide.

The fine structure of polyacrylamide gels prepared with cross-linkers other than bisacrylamide[35–38] was also examined. Cross-linkers such as DATD allow the gel to be dissolved in periodic acid for high-yield recovery of proteins. These cross-linkers do not display increasing opacity at higher values of C as do bisacrylamide-containing gels. This difference can be demonstrated by TEM, which shows that gels cross-linked by bisacrylamide display increasingly larger structures as the crosslinker is increased above 5% (Fig. 3). These structures interfere with visible light. DATD-crosslinked gels of the same composition do not show such structural changes (Fig. 5). Structural observations suggest that highly cross-linked DATD gels cause more sieving of macroions than comparable gels containing bisacrylamide, this has been confirmed empirically[28]. The difference between these two types of gel involves their polymerization properties. Pure solutions of DATD do not polymerize, whereas bisacrylamide polymerizes easily into a white rigid mass of clustered structures (Fig. 6). These results correspond to findings with the SEM[9] and confirm theoretical considerations[17]. The clustered appearance of pure bisacrylamide gel is accompanied by increased hydrophobicity of the gel. Thus gels of 5% T and 50% C can be transferred, after polymerization in water, to chloroform–methanol without shrinkage or loss of flexibility, whereas gels of 20% C or less shrink and become rigid upon such a transfer[15].

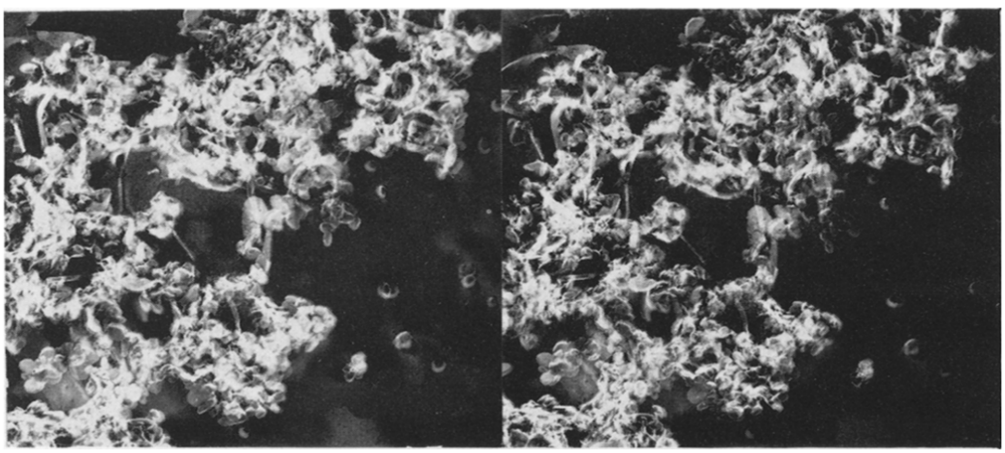

Fig. 6. TEM stereo image of poly-bisacrylamide (1.7%, w/v; without acrylamide); the bar equals 400 nm. Poly-bisacrylamide is a white rigid structure that displays an unorganized clustered substructure. Such gel does not swell in water and can be transferred to organics.

An alteration in gel morphologic structure is caused by cross-linkage with a disulphide-containing analogue of bisacrylamide (bisacrylylcystamine), which allows gels to be dissolved in solutions containing reducing agents at physiological pH[38]. These gels can be polymerized only above *ca.* 3% (w/v) acrylamide concentration, they display a structure that is dominated by evenly distributed round pores (Fig. 7).

Polymerized acrylamide without cross-linkage is shown in Fig. 8; this polyacrylamide remains in the sol state and yields a solution of very high viscosity that may be useful for stabilizing density gradients. Uncrosslinked polyacrylamide has recently been used in combination with an agar gel matrix as a sieving medium for

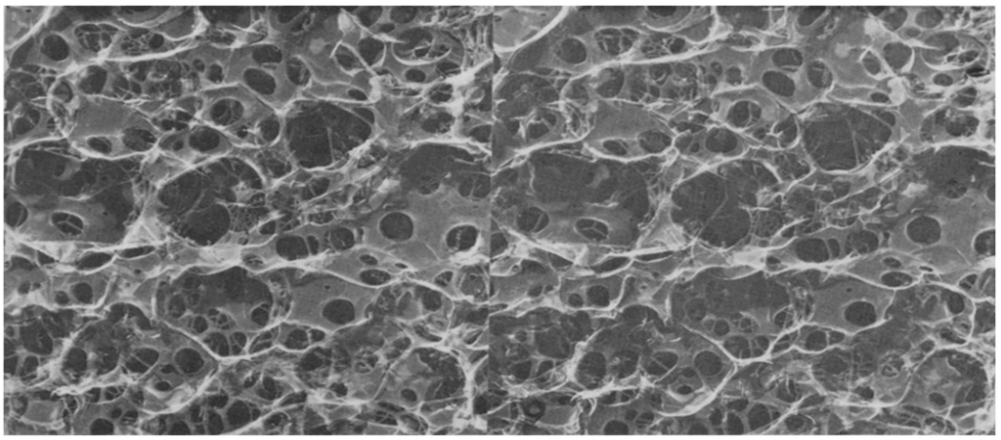

Fig. 7. TEM stereo image of 5% T, 5% C polyacrylamide gel that is cross-linked by acrylylcystamine; the bar equals 400 nm. The disulfide bond-containing cross-linker changes the overall structure of the gel matrix as compared with a gel containing bisacrylamide (Fig. 3) or diallyltartardiamide (Fig. 5). Round holes of a fairly even size in the gel membranes are a specific feature of this type of gel.

electrophoresis[39]. Uncross-linked polyacrylamide displays elongated parallel superstructures, a feature that agrees with previous findings with SEM[9] and correlates with the longitudinal bundles of fibres predicted by Flory[14].

Recently, several investigators have discussed the possibility that varying physical conditions during polymerization influence the properties of polyacrylamide gels. Polymerization temperature affecting the speed of polymerization as well as the sieving properties of the gel has been described[40]. Altering the polymerization speed by varying the catalyst concentration, which was increased two times or reduced by 50%, did not cause consistent changes in the spongelike structure of the gel.

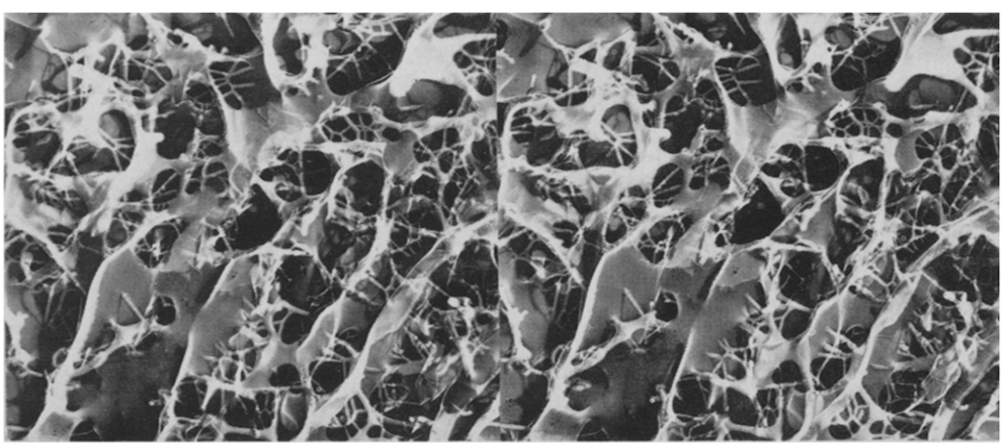

Fig. 8. TEM stereo image of polyacrylamide (5%, w/v), without cross-linker); the bar equals 400 nm. Uncross-linked polyacrylamide is a viscous syrup, not a gel but in the sol state, and shows a variable, porous structure with some organization into parallel leaflets. Polyacrylamide is very hydrophilic and has a strong tendency to swell in water.

This observation, which is also true for a change of the ratio of Temed and persulfate, confirms earlier results obtained with SEM. Therefore, alterations must be traced back to the substructure of the gel membranes.

A quantitative evaluation of the gel structures related to changes of total acrylamide concentration (T) and cross-linkage (C) requires a statistical approach possibly including automated scanning of electron micrographs. Because this has not been done, a comparison of theoretical implications derived from a gel model employing a random meshwork of fibers[21,41,42] with data based on the evidence of fibrous membranes that are divided by free spaces has not been attempted.

Also, we have not yet been able to examine the matrix structure of polyacrylamide gel that has been polymerized in a cryoprotectant such as glycerol or glycol. Thus, the possibility of artifacts caused by freezing cannot be totally discounted.

The results obtained thus far by electron microscopy are not unequivocal[7]. This is not surprising since a structure like that of the gel matrix, whose function is dependent on hydration, is not likely to yield easily when dehydration is required for its investigation. Nevertheless, the demonstrated relationship between structural features of the gel matrix and data concerning the optical and electrophoretical properties of the gels suggests that the structures illustrated in this paper are at least derived from the real sieving structure of polyacrylamide and are not due to artifacts resulting from freezing or dehydration.

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