ON THE INTERDEPENDENCE OF PROTEIN DIFFUSION, ELECTROPHORETIC MOBILITY AND GEL STRUCTURE

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(Received September 3rd, 1959)

Since GORDON, KEIL AND ŠEBESTA[1] succeeded in 1949 in fractionating proteins in a 1 % agar gel, this support has found a wide application for electrophoretic work. When electrophoresis is followed by gel diffusion of the specific antibody we have immuno-electrophoresis, as developed by GRABAR AND WILLIAMS[2] in 1953; the method has become a valuable tool for clinical and immuno-chemical investigation, since it possesses the increased sensitivity of the immunological techniques. Using double diffusion of antigen and of antibody in an agar gel[3], the presence or absence of immunologically specific antigen antibody systems can be demonstrated. Agar gel electrophoresis found new applications: GIRI[4] introduced a two-dimensional technique and WIEME AND RABAEY[5] developed ultramicroelectrophoresis on a quantitative basis; the latter gives good resolution starting with 0.1 ml of a protein solution of 0.02 % (0.02 mg of protein). Other studies[6] have reduced the agar concentration to a point just below its gelling concentration. Since an agar gel of 1 % still shows sufficient rigidity, 99 % of the gel is fluid and allows for conditions which equal those of "free" electrophoresis; in comparison with starch as a support medium, agar has the disadvantage of a higher adsorption for proteins and a strong electroosmotic flow.

Though numerous symposia[7] have collected reports of investigations on diffusion and immunological reactions in agar gel, only a few papers[6,8] have dealt with the colloid-chemical side of these phenomena. The aim of the present contribution is to demonstrate the correlation of gel structure with diffusion and electrophoretic mobility of proteins. In particular, the influence of carbohydrates on the structure of the agar gel is dealt with.

METHODS

(1) *Gel diffusion*

On glass plates of 4 × 16 cm, as used with the Elphor-apparatus for paper electrophoresis, a gel is formed with 12 ml of 1.5 % agar (Difco Bacto Agar) in veronal acetate buffer of pH 8.6 and $\tau/2 = 0.1$. On this gel—the basis gel—an intermediate gel of 1.8 mm thickness is laid[9]; it contains 0.666 % agar and various carbohydrates capable of influencing gel structure (see Table II). In order to estimate the correlation of molecular weight and particle size with diffusion velocity, three protein solutions containing 2 % of serum albumin* (human), 2 % γ-globulin* (human) and α_2-macro-

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globulin*, are prepared. These solutions are taken into the Agla pipette; 6–7 drops containing 200 μg of a fraction are placed at regular intervals on the surface of the intermediate gel, which has been prepared 1 h previously. After 48 h in a wet chamber at 20° these intermediate gels are gently stripped off and laid on a glass plate of 4 × 16 cm. Together with the basis gels they remain for 3 h in 2 % acetic acid; later they are covered with a wet filter paper, dried under infrared light and stained with Naphthalene Black 12B 200[10]. Two gel plates are always run in parallel; these allow for 12–14 stained spots of protein, which are then measured in the Zeiss Extinktionsschreiber II; the smallest and the largest values are attributable to gel heterogeneities and are ruled out; the remaining values must not differ by more than 5 % (see Fig. 1).

(2) *Electrophoresis*

On glass plates of 4 × 16 cm a gel is formed with 12 ml 1.25 % agar (0.19 ml/cm^2; thickness of gel 1.9 mm) in the above-mentioned buffer of pH 8.6. All the runs are made in duplicate and last 6 hours. The current is switched on 30 min before the proteins are placed on the gel. A mixture is prepared containing 2 % serum albumin and 2 % γ-globulin; a drop from the Agla pipette, containing 200 μg of each fraction, is placed on the centre of the agar gel. After an interval of 15 min to allow the liquid to diffuse into the gel, the current is switched on. The voltage is stabilized by the Reco model E-800-2** Powerstat. In order to determine the electroosmotic flow, a drop of a 16 % solution of PVP (Plasdone) is placed on the centre of the agar gel. After the run this gel is covered with a strip of moist filter paper Munktell 20/150 and dried under the infrared lamp; the paper takes up the PVP. It is then dyed for 5 min with bromophenol blue and washed with water, when the position of the PVP spot is revealed. The distance travelled by the PVP spot from the starting line is added to the distance of protein anions and subtracted from the cations; thus μ is arrived at in $cm^2 \cdot sec^{-1} \cdot V^{-1}$. The proteins in the agar film are stained for 10 min in a solution of methanol, saturated with Naphthalene Black 12B 200 and containing 10 % acetic acid. Subsequent washing is carried out with methanol + 10 % acetic acid, changed three times. This technique allows for *independent measurements of electrophoretic migration velocity as well as electroosmotic flow in the same run.*

(3) *Specific conductance*

The resistance v of the agar gel between the electrodes is expressed by the equation

$$v = \frac{V}{A} = \sigma \cdot \frac{l}{q}$$

where l is the length between the electrodes, 12.5 cm, and q the surface of a cross-section, 0.072 cm^2. The specific conductance $\varkappa$ equals the reciprocal specific resistance σ.

$$\varkappa = \frac{A}{V} \cdot \frac{l}{q} = \text{Ohm}^{-1} \cdot \text{cm}^{-1}$$

* Behring Werke, Marburg/Lahn, Germany
** Research Equipment Corp., Oakland, Calif.

with the current intensity A in mA and the voltage in V. Electrolytical conductivity is generally an exponential function of the absolute temperature; therefore all runs were made at a constant temperature of 20°.

(4) *Colloid chemistry of the gel*

(*a*) *Viscosity*. All measurements were done with the Ostwald capillary viscosimeter in a constant temperature bath at 70°. In order to correlate the specific viscosity η_{sp} with concentration C, η_{sp} is divided by C. The resulting viscosity number or index η_c is then

$$\eta_c = \frac{\eta - \eta_0}{\eta_0 \cdot C} = \frac{\eta_{sp}}{C}$$

(*b*) *Turbidity* is measured in the Zeiss Step Photometer with a special attachment for the measurement of stray light under an angle of 45°. The sols were measured at 70°, the gels at 20° 2 h after setting. The turbidities are given in percentages of the standard turbidity No. 4, which is taken as 100; our data are thus relative turbidities but comparable.

(*c*) *Tarr–Baker test*. This test was developed in 1926 for testing "jelly strength"[11–14]. A cylindrical glass stamp of 20 mm diameter is slowly pressed into the gel; when the ultimate strength is passed, the surface cracks and the stamp breaks into the gel. The critical pressure is given by the length of a CCl_4-column in cm. This length multiplied by 1.6 gives the length of a corresponding water column in cm (see Table III). Gels are tested 7 h after they have set.

(*d*) *Säverborn test*. By this test the rigidity of the gel can be measured[15,16]. The gel is formed in the space left between an inner and an outer metallic cylinder. On the former a constant force is acting to turn the inner cylinder in the gel. The counteraction of the gel gives a measure of its torsional strength; it is measured optically and expressed in dynes per cm^2 (modulus of rigidity). Gels are measured 7 h after they have set[17–19] at a temperature of 18°. The gel is covered in order to minimize evaporation.

RESULTS

Diffusion

In order to obtain comparative values of stained proteins without diffusion, 7 drops containing 200 μg of serum albumin were placed by means of the Agla pipette on a 1.5% agar gel 1.8 mm thick. Two hours later the gel was dried and stained with Naphthalene Black. The same was done with γ-globulin and with α_2-macroglobulin. For each protein 20 spots were measured by the Zeiss Extinktionsschreiber II and the results compared statistically ($n = 20$).

For the following studies of gel diffusion the surfaces quoted in Table I, column 4, are standard values (100%); the surfaces found after diffusion through the intermediate gel (*cf.* METHODS (1)) are given in percentages of the standard (Table II). In Fig. 1 the surfaces of stained spots of protein are depicted as they are delivered by the Zeiss apparatus.

Contrary to the experience on cellulose[22,23] γ-globulin takes a slightly more intense stain (Naphthalene Black 12B 200) than serum albumin.

The colloid-chemical data of these intermediate gels are given in Table III. Early electrophoretic measurements had shown that the results in 0.666 %

TABLE I

Protein fraction	*Mol. wt.**	*Length and diameter** Å	*Diffusion constant* $D_{20} \times 10^{-7}$ *cm²/sec*	*Surface of stained spots (average value)* cm^2	*Standard deviation* σ	*Deviation of the average value* $\sigma/\sqrt{n}$	*Average error* $\sigma^2/\sqrt{n}$
Serum albumin	69,000	150 × 38	6.1 [25]	1.15	± 0.060	0.0166	0.00028
γ-Globulin	152,000	235 × 44	3.6 [26]	1.60	± 0.076	0.0197	0.00038
α_2-Macroglobulin[24]	846,000	—	—	0.75	± 0.074	0.0208	0.00043
Plasdone** (PVP)	90,000 ± 15,000	350 × 18	—	—	—	—	—

* From sedimentation and diffusion measurements[20,21].
** Antara Chemicals, New York, N.Y., U.S.A.

TABLE II

Intermediate agar gel of 0.666 %	*Diffusion into basis gel after 48 h*		
	Albumin %	*γ-Globulin* %	*α_2-Macroglobulin* %
Agar gel, 0.666 %	47	38	30
Additions:			
3.33 % Sucrose	65	43	38
3 % Dextran L*	42	40	15
0.83 % Dextran H*	19	30	trace
0.025 % Carrageenan**	9	19	trace

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** Institute for Agricultural Chemistry of the Federal Polytechnical School, Zürich (Prof. Dr. H. DEUEL).

TABLE III

Gel composition	*Mol. wt. of additions*	*Viscosity* η_{sp}	*Turbidity rel.%*		*Tarr-Baker test (gel strength) cm H_2O column*	*Säverborn test (rigidity) dynes/cm²*
			Sol	*Gel*		
Agar gel, 0.666 %	—	0.76	21	225	138	$6.4 \cdot 10^3$
Additions:						
16.66 % Ethylene glycol	62	1.58	17	178	160	$7.1 \cdot 10^3$
3.33 % Sucrose	349	1.19	16	185	170	$6.3 \cdot 10^3$
3.00 % Dextran L	15,000– 20,000	1.18	20	250	159	$11.0 \cdot 10^3$
0.83 % Dextran H	90,000–100,000	1.38	60	232	159	$7.6 \cdot 10^3$
0.10 % Carrageenan	approx. 300,000	3.29	18	192	216	$10.8 \cdot 10^3$
0.025 % Carrageenan		1.57	14	138	181	$8.6 \cdot 10^3$

agar-gel, supplemented with the additions indicated in Table II, do not reveal significant changes in migration velocity. Therefore the following experiments were carried out in 1.25 % agar gel, with a field strength of 1.60 V/cm and a current intensity of

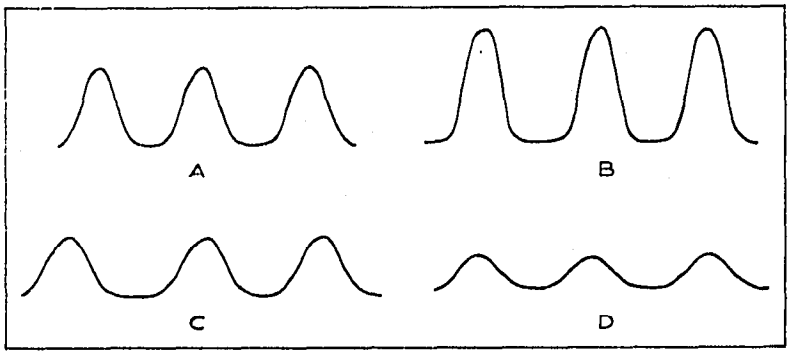

Fig. 1. Curves of stained protein spots before and after diffusion, as given by the Zeiss Extinktionsschreiber II.

2.25 mA/cm. Duration of electrophoresis was 6 h. The temperature in the gel did not rise above 20°. Evaporation was minimal since the gels were covered with a sheet of wet porous rubber[6]; their loss in weight remained below 4 %.

Gels containing ethylene glycol and Dextran H adhered so strongly to the paper, when drying, that the staining was much impaired.

Since the addition of 0.10 % Carrageenan to agar gel causes extremely high values for viscosity, gel strength and rigidity (see Table III), the rate of electrophoretic migration was measured in gels of 1.25 % agar in a buffer of pH 8.6, $\tau/2 = 0.1$ and simultaneously in gels of 1.25 % agar plus 0.1 % Carrageenan. These gels were formed on glass plates 10 × 15 cm; they contained 0.24 ml/cm² and were 2.4 mm thick. The size of the gel allowed 6 electrophoretic runs under strictly equal conditions of temperature and evaporation. Each run was done three times; reproducibility of results lies within ± 5 %. The field strength was kept constant at 2.26 V/cm and the current intensity at 4.00 mA/cm. The rate of migration and of electroosmosis was determined after 2, 4 and 6 hours.

TABLE IV

ELECTROPHORETIC DATA FOR ALBUMIN AND γ-GLOBULIN

Gel	*Electroosmosis cm*	*Distance of separation cm*	*Corrected length of migration cm*		*Rate of migration in cm/min × 10⁻²*	
			Alb.	*γ-Glob.*	*Alb.*	*γ-Glob.*
Agar gel, 1.25 %	2.3	3.3	+ 3.6	+ 0.3	+ 1.00	+ 0.08
Additions:						
3.33 % Sucrose	1.6	3.7	+ 2.8	— 0.9	+ 0.77	— 0.25
3.00 % Dextran L	1.7	3.2	+ 2.7	— 0.5	+ 0.75	— 0.14
0.025 % Carrageenan	1.4	3.7	+ 3.0	— 0.7	+ 0.83	— 0.19
0.100 % Carrageenan	1.2	3.7	+ 2.7	— 1.0	+ 0.75	— 0.27

+ anodic migration. — cathodic migration.

TABLE V

Duration h	1.25% Agar gel				1.25% Agar gel + 0.10% Carrageenan			
	Electro-osmosis (PVP) cm	Corrected rate of migration in cm/min × 10^{-2}			Electro-osmosis (PVP) cm	Corrected rate of migration in cm/min × 10^{-2}		
		Alb.	α_2-Macro-globulin	γ-Globulin		Alb.	α_2-Macro-globulin	γ-Globulin
2	1.3	1.83	1.47	0.44	0.6	1.52	0.73	0.28
4	2.8	1.87	1.50	0.46	1.2	1.54	0.76	0.29
6	4.2	1.86	1.50	0.44	1.9	1.52	0.75	0.29
Average values		1.85	1.49	0.45		1.53	0.75	0.29

DISCUSSION

Comparison of the results of diffusion in Table II shows that protein diffusion through 0.666% agar gel is limited; after 48 h the percentage values for serum albumin, α_2-macroglobulin and γ-globulin are 47, 38 and 30, respectively. This slowing down by gel porosities is accentuated by the following additions of carbohydrates. α_2-Macroglobulin is slowed down by the addition of Dextran L (mol.wt. 15,000–20,000); the same applies to albumin and to γ-globulin if Dextran H (mol. wt. 90,000–100,000) or Carrageenan (mol. wt. approx. 300,000) are added to the agar gel. In both these cases gel diffusion of α_2-macroglobulin is almost entirely stopped. Carrageenan is a branched chain polysaccharide from seaweed, composed chiefly of D-galactose residues[27,28]; it increases the viscosity, gel strength and rigidity of the agar gel (see Table III) without chemical reaction (gel turbidity remains low). The increase in the density of the network seems to be independent of the linear D-galactopyranose residues of agar (see Fig. 2).

Thus the change in gel structure is different from the increased α-1,6-glucosidic cross-linking of dextran used for the purpose of gel filtration[29]; here branch units, which normally occur only for every 20 glucose residues, are multiplied by chemical

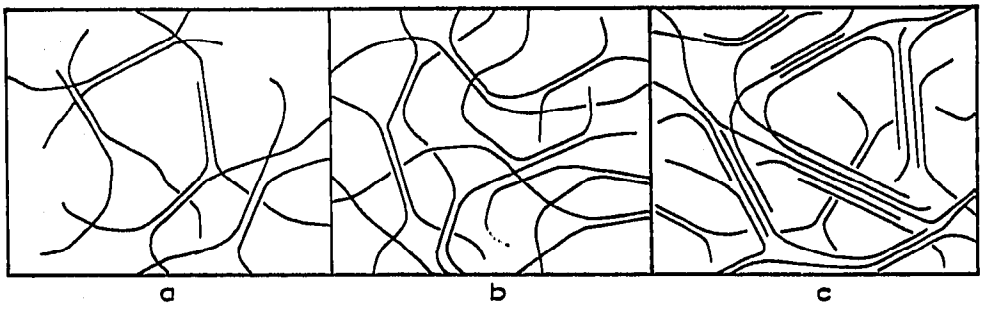

Fig. 2. Schematic representation of network of linear colloids. (a) Agar gel. (b) Agar gel + Carrageenan. (c) Dextran with specially increased cross-linking.

reaction. As a result the dextran gel develops from a linear colloid into a three-dimensional network. The agar/Carrageenan combination brings about no molecule sieve either; γ-globulin diffuses more freely than albumin, a fact which indicates

appreciable heteroporosity. The effective pore diameter of a 2 % agar gel is given by TSENG[30] as approx. 30 Å; in this case diffusion would be limited by substances with a molecule radius of more than 2 Å (urea $\nu = 2.7$ Å; sucrose $\nu = 4.4$ Å). If we find that 47 % of the albumin has diffused through the 0.666 % agar gel within 48 h and if we assume that the diffusion rate has been lowered to 50%, then we can deduce that there must be pores with $\nu = 120$ Å[31]. These would also allow γ-globulin to pass through. As for α_2-macroglobulin, no estimates can be given since length and diameter have not been stated, but it can be deduced from the molecular weight, which is twelve times that of albumin, that a considerably wider pore size must exist. Thus it appears that the 0.666 % agar gel is highly heteroporous. The study of percentage distribution of pore sizes is complicated by the fact that the diffusion rate remains equal if the gel membrane contains per cm^2 a single pore of $\nu = \sqrt{\pi^{-1}}$ cm or 10^4 pores of $\nu = 10^{-2} \cdot \sqrt{\pi^{-1}}$ cm each. Addition of sucrose generally causes an increase in porosity, whereas addition of Dextran H and of Carrageenan apparently decreases the average pore size; but the results given in Table II convey the impression that large pores fall out earlier than medium-sized pores. As has been stated by MCDONALD[32] for electrophoretic migration in paper, it seems very possible that *different migrants follow different migration paths in gel diffusion*; this would apply particularly in the case of large molecules where intrinsic viscosity[33] and the diffusion constant vary strongly. In our case $[\eta]$ for serumalbumin is [0.034] and for γ-globulin [0.064–0.066] and $D_{20} \times 10^{-7} = 6.1$ and 3.6, respectively (see Table I). The intrinsic viscosity is a measure of the contribution of the individual protein molecule.

As has been stated already, it was necessary to increase the agar concentration in order to get significant differentiation of electrophoretic migration velocity. Suitable gels must contain 1.25 % of agar; unfortunately, their rigidity is such that measurements of viscosity and gel strength are not possible. Though the measurement of small moduli has become possible lately[34], it is still impossible in the case of gels with a high ratio of shearing stress to strain. We did not try to decrease electroosmosis by purification of the Bacto-agar (WIEME[35]); the important part played by electroosmosis would be less perceptible. To inhibit it altogether by addition of 2 % of gelatinized starch[36] would change all the colloid-chemical factors.The addition of 3.33 % sucrose shows a 30 % decrease of electroosmosis (see Table IV), which is in accordance with the findings of PREER AND TELFER[37]. On addition of 0.1 % Carrageenan the decrease in electroosmosis amounts to 48 %; all migrants are slowed down accordingly (see Table IV). But on the addition of dextrans the effect is much the same, despite the fact that colloid-chemical values differ significantly. In early attempts at electrophoresis with a 1 % agar gel as support[38], the migration of haemocyanin with a mol. wt. of 8,500,000 was recorded. Later studies[8] with "Levan" of mol. wt. 1000 showed unhindered migration in a 2 % agar gel, whereas "Levan" of mol. wt. 1,000,000 was slowed down by 75 %. Our experiments with α_2-macroglobulin of mol. wt. 846,000 show a decrease in the rate of migration of 50 % when 0.1 % of Carrageenan is added to the 1.25 % agar gel. With albumin the decrease amounts to 17 % and with γ-globulin to 35 % (see Table V). Here then *a correlation of molecular size and volume*

with the rate of electrophoretic migration seems to exist (*cf.* [39,40]). The example of fibrinogen, with a mol. wt. of 400,000 and a molecule thickness the same as that of albumin, which does not move from the starting line even in 0.666 % agar gel, is proof of the importance of the chemical nature of the end groups. Even purified agar specimens contain between 0.3 and 3.7 % sulphur. It is probable that the resulting acidic nature promotes the resolution of normal adult haemoglobin into fractions; if paper is used as support it shows a homogeneous protein. Also in our experiments (Table V) these acidic groups in the agar gel alter the electrokinetic conditions considerably, whereas diffusion of proteins is much more sensitive to alterations in the pore size of the agar gel network. Thus a correlation of systematically altered colloid-chemical gel conditions and electroosmotic flow is readily visible but is not paralleled by changed electrophoretic resolution or separation of protein fractions.

ACKNOWLEDGEMENTS

Our thanks are due to Prof. H. DEUEL and Dr. J. NEUKOM, who provided us with apparatus.

SUMMARY

In order to study the possible correlation between protein diffusion, electrophoretic mobility and agar gel structure, the last-mentioned factor has been characterized by means of colloid-chemical methods (viscosity, turbidity, gel strength and rigidity).

Gel diffusion of serum albumin, γ-globulin and α_2-macroglobulin was measured. New limits of diffusion resulted when dextran or Carrageenan were added to the agar gel. Such additions of carbohydrates decreased electroosmosis and tended to slow down the rate of migration. The interdependence is discussed.

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