Demonstration of Molecular Size Differences Between Closely Related Antigens by a Simple Gel Diffusion Test

The size and shape of a protein molecule has been recognized as a major determinant of diffusion rate in single or double diffusion procedures in gels[1]. ALLISON and HUMPHREY[2,3] carefully studied the application of a double diffusion test and of the molecular sieve effects of concentrated gels to molecular weight determination of proteins. Recently their principles have been adapted to the detection of 7S IgM by STOBO and TOMASI[4] and SOLOMON[5].

It is known that IgM diffusibility in gels increases after depolymerization with thiols[6–9]. During our studies on the action of DL-Penicillamine and other thiols on immunoglobulins (results to be published), a simple diffusion test was developed with the aim of allowing an easy demonstration of the molecular size changes induced by the treatment. This test is easily adaptable to other purposes and only standard materials are required to its performance.

Materials and methods. Samples of whole serum containing monoclonal components[10] immunologically classified as IgG or IgM and isolated monoclonal components were submitted to the action of 0.05*M* DL-Penicillamine (DL-*P*)[11], 0.05*M* alpha-mercaptopropionylglycine (α-MPG)[12], 0.005*M* Dithiothreitol (DTE)[11] and 0.1*M* 2-mercaptoethanol (ME)[13] for periods of time respectively of 18 h (overnight) for DL-*P* and α-MPG, 2 h for DTE and 15 min for ME, and in all cases reduction took place at room temperature. A 10% excess of iodoacetamide[13] was added after the reduction reached its end. Reducing and alkylating agents were added to the serum in the form of 1 part of an adequate solution, prepared in 0.2*M*, pH 8.0 *Tris*-HCl buffer, to 4 parts of serum. Control samples of each treated serum or preparation were obtained adding 2 parts of this buffer to 4 parts of serum.

A sample of normal IgG, isolated by batch chromatography in DEAE[14,15] was digested with papain[16], according to PORTER[17]. For subsequent studies 5 mg/ml solutions of the isolated IgG and of the digest were utilized.

Comparative immunodiffusion test. For the immunodiffusion studies 7 × 7 cm glass plates were covered with a layer of 12 ml of 2% agar[18] in 0.3*M* phosphate buffer, pH 8.0.

Holes (2 mm diameter) and troughs (2 × 60 mm) were cut in the agar in a similar arrangement of one of those described by ALLISON and HUMPHREY[2,3], as shown in Figure 1. Control and depolimerized samples were arranged as shown in Figure 2.

The troughs were filled with the appropriate antisera, either absorbed so as to be specific for the heavy chains of IgG and IgM[19] or a mixture of 3 monospecific antisera (anti IgG, IgA and IgM) in a proportion which gave 3 distinct lines in Ouchterlony analysis or immunoelectrophoresis. When studying the effects of papain digestion on the diffusion rate, anti whole IgG was used.

Diffusion was allowed to proceed for 3–4 days at 4 °C, and then the plates were washed and stained with Amidoblack[20].

On the stained plates the distance from the nearest point of each precipitation arc to the trough (Figure 2) was determined to within 0.1 mm with the aid of a magnifying glass with a graduated scale[21]. The average of the triplicate measurements obtained for control and depolymerized samples of each serum was calculated, and the difference between the averages was determined.

Gel filtration on Sephadex G-200[22] was performed on a 40 cm column. 0.5–1 ml samples of control and treated samples were studied. 0.4*M* *Tris*-HCl buffer, pH 8.0 was used with a flow rate of about 6 ml/h. 20 min samples were collected on a fraction collector and immunologically analysed.

Results and discussion. In Figure 2 is shown the typical result of the comparative study of control and thiol-treated samples from a serum containing an IgM monoclonal component. The shift of the precipitin lines towards the trough, its thickening, and the lack of protein retention around the wells are the main changes observed on thiol-treated samples when compared with their correspondent controls. When an anti-mixed immunoglobulin antiserum is placed in the trough (Figure 3), the

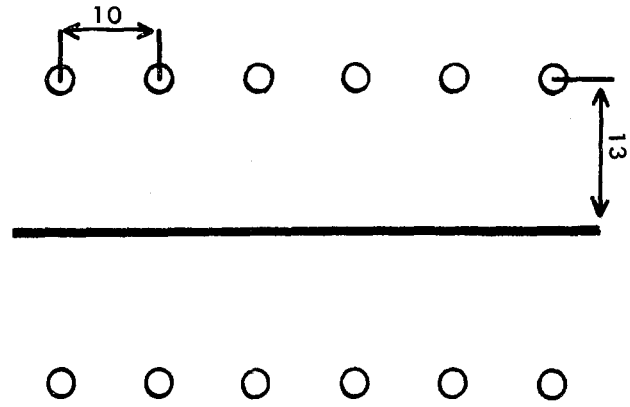

Fig. 1. Arrangement and distances between well and trough (in mm) in the comparative immunodiffusion test.

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[11] Sigma Chemical Co., St. Louis (USA).
[12] Offered by Calbiochem. Ltd., London (England).
[13] L. Light and Co., Colnbrook (England).
[14] Whatman DEAE 52, W. and R. Balston, Springfield Mill, Kent (England).
[15] D. R. STANWORTH, Nature *188*, 156 (1960).
[16] Sigma Chemical Co., St. Louis (USA).
[17] R. R. PORTER, Biochem. J. *73*, 119 (1959).
[18] Difco Labs., Detroit (USA).
[19] All the specific antisera were prepared in the Department of Experimental Pathology, University of Birmingham (England).
[20] G. T. Gurr, London (England).
[21] Flubacher and Co., Horgen (Switzerland).
[22] Pharmacia Fine Chemicals, Uppsala (Sweden).

only affected precipitin line is the one corresponding to IgM.

Ten sera containing monoclonal IgM and ten sera containing monoclonal IgG were analyzed. The differences between the distances from the precipitin arcs to the trough obtained from control and treated samples of each serum are shown in the Table. For IgM these usually ranged from 2.2 and 2.8 mm, contrasting to a maximal difference of 0.2 mm recorded for an IgG monoclonal component. Repeated analyses of a single IgM containing serum depolymerized with the same thiol produced results comprised between $\pm$ 0.2 mm to the mean.

Sephadex G-200 gel-filtration studies were performed in the samples indicated in the Table. Figure 4 shows the result of the immunological study of the pooled and concentrated fractions obtained from the control and DL-*P* treated samples of serum MM19. Similar results, showing that the bulk of IgM is present in the fractions of the first peak (19S) of the control sample, and in the fractions of the second peak (7S) of the thiol-treated sample, were found in the other cases studied. This confirms the usefulness of this test for assessing the effect of thiols on IgM. Shifts ranging from 2.2–2.8 mm were characteristic of the split of 19S IgM into its 7S subunits, and the test allows a rapid and simple screening of the

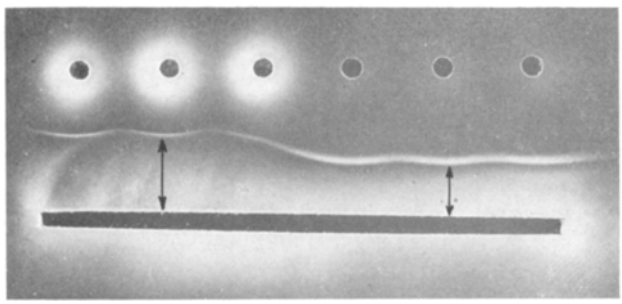

Fig. 2. Comparative immunodiffusion study of the control samples placed on the 3 wells to the left of the middle line, and the DTE-treated samples, placed on the 3 other wells, of serum MM7. The trough was filled with anti-IgM antiserum. The arrows illustrate the method of measurement of distances from the nearest point of each precipitin arc to the trough.

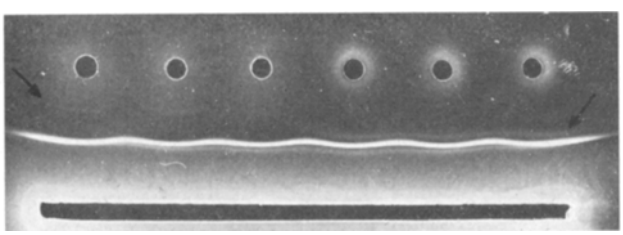

Fig. 3. Comparative immunodiffusion study of 3 control (left-sided wells) and three DL-*P* treated samples of the same IgM containing serum with anti-mixed immunoglobulin antiserum. The IgM arc is indicated by arrows.

Differences (in mm) between the distance from the antiserum trough to the precipitation arcs corresponding to control and thiol-treated samples of 10 sera containing IgM monoclonal components and 10 samples of sera containing IgG monoclonal components

IgM monoclonal gammopathies treated with Serum	DL-*P*	α-MPG	DTE	ME	IgG monoclonal gammopathies treated with Serum	DL-*P*	α-MPG	DTE	ME
	ref.					ref.			
MM3	2.3	2.3	–	–	MG1	0	–	–	0
MM7	2.6[a]	–	2.6	–	MG2	–	0.1	0	–
MM9	2.7	–	–	2.5	MG6	0	0	0	–
MM10	2.8[a]	2.6	2.8	2.9	MG7	0	–	–	–
MM11	2.4	2.4	2.3	–	MG8	0	0.1	0	–
MM12	2.3	2.1	–	–	MG10	0.1	–	–	–
MM16	2.3	2.6	2.3	–	MG14	0	–	0	–
MM17	2.6[a]	2.3	2.4	–	MG19	0	–	–	0
MM19	2.6[a]	2.4	–	–	MG32	–	0.2	0.2	–
MM22	2.8	2.4[a]	2.7	–	MG33	0	–	–	–

[a] Indicates Sephadex G-200 study of that serum.

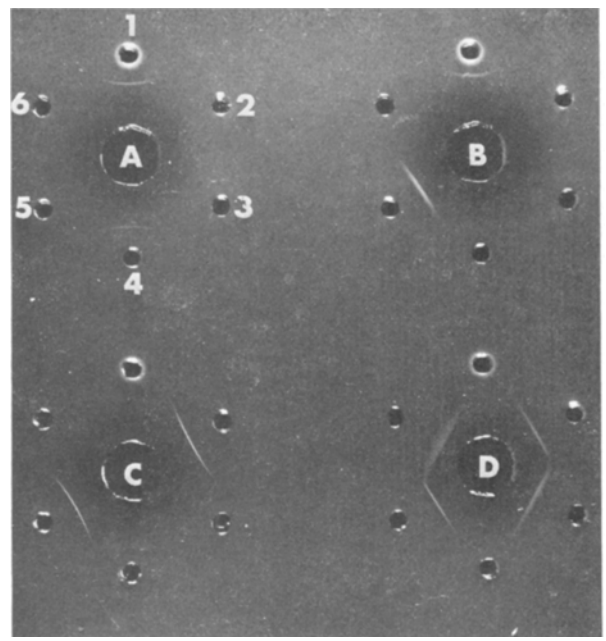

Fig. 4. Immunodiffusion study of the pooled and concentrated fractions obtained by Sephadex G-200 gel filtration of a control and a DL-*P* treated sample of serum MM19. The arrangement is the same in the 4 systems: control sample – wells 1 (first peak), 2 (second peak) and 3 (third peak); DL-*P* treated sample – wells 4 (first peak), 5 (second peak) and 6 (third peak). The following antisera were used: anti-alpha-2-M (A), anti-IgM (B), anti-IgG (C) and anti-albumin (D).

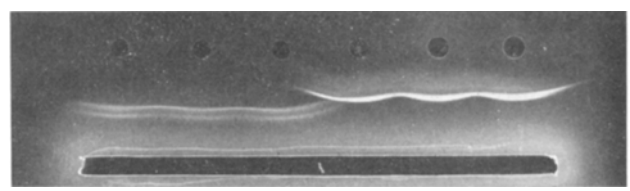

Fig. 5. Immunodiffusion study of 3 IgG samples, 5 mg/ml (right-sided wells) and 3 samples of the papain digest of the same IgG, 5 mg/ml, with anti-whole IgG antiserum.

results of treatment with thiols of a large number of samples.

Theoretically this comparative immunodiffusion concept may be applied for the detection of molecular size differences between any proteins able to be precipitated by the same antiserum. In Figure 5 is shown the study through this test of a control and a papain-digested solution of IgG. Not only the increased diffusibility of the digest products but the difference in diffusion rate between Fab and Fc fragments are shown.[23]

Résumé. Des différences dans les dimensions moléculaires de protéines possédant des spécificités antigéniques communes sont traduites par des différences dans la diffusion en gels d'agarose. Ces différences sont facilement mises en évidence par un test de double immunodiffusion en gel d'agarose, en employant un arrangement spécial des échantillons antigéniques et de l'antiserum.

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Electron-Microscopic Study of Antibody-Producing (Plaque-Forming) Spleen Cells of the Tortoise *Agrionemys horsfieldi* Gray

In the past few years the hemolytic plaque technique has been adapted to electron-microscopy, thus providing a possibility to study the ultrastructure of antibody-forming cells[1-4]. So far antibody-producing cells of poikilothermic vertebrates have not been subjected to ultra-structural analysis. The technique, originally described for mammalian cells, was used to study spleen cells of the tortoise. The kinetics of both antibody formation[5-9] and the production of plaque-forming cells[10] in the tortoise have been described recently. These cells of *Agrionemys* (Testudo) *horsfieldi* Gray have an average diameter of 10 µm and an eccentric nucleus.

The ultra-structure of plaque-forming cells of the tortoise shows great variation. Some cells have a comparatively primitive cytoplasmic morphology with a slightly developed endoplasmatic reticulum, numerous ribosomes, while only few adhere to the endoplasmatic reticulum, and an almost spherical nucleus (Figure 1). In all types of plaque-forming cells there are some large mitochondria. In cells most frequently observed, an endoplasmatic reticulum with typically extended cisterns may be noted. The nucleus has an irregular shape (Figure 2). There are intermediate stages between these 2 cell-types. Up to today no antibody-producing cell with the characteristically layered ergastoplasm of the

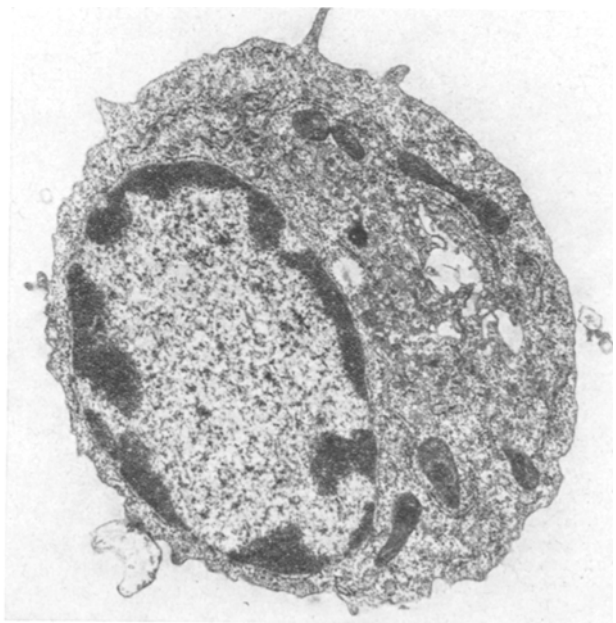

Fig. 1. A plaque-forming cell of tortoise with a comparatively primitive morphology of cytoplasm. × 20,000.

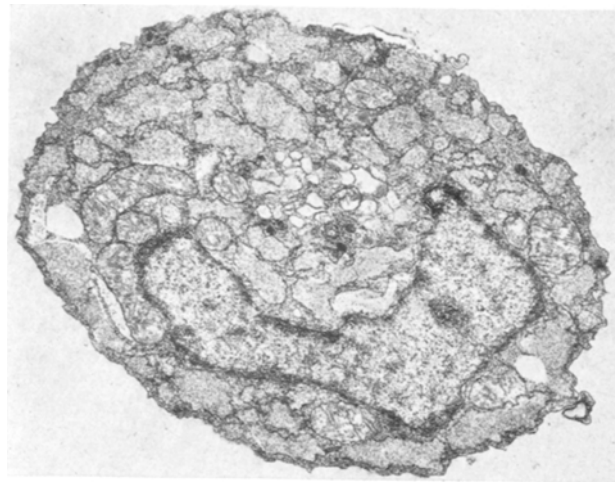

Fig. 2. An antibody-producing cell of tortoise with extended endoplasmic cisterns. × 20,000.

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