Handbook of Immunodiffusion and Immunoelectrophoresis

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(with appendices by three other authorities, and an equipment section)

Contents

IMMUNODIFFUSION AND IMMUNOELECTROPHORESIS

BIBLIOGRAPHY

FOUR APPENDICES

Diffusion-in-gel Methods for Immunological Analysis

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From PROGRESS IN ALLERGY

INTRODUCTION

During the years which have passed since the last survey in this series on diffusion-in-gel methods for immunological analysis was published by OUCHTERLONY (158), these techniques have been increasingly applied to problems in various fields of research. Further modifications of the techniques have also been elaborated. Short surveys on the technical development of immunodiffusion methods and their application have been presented during the last few years by, *e.g.* GENDON (65), BOYD (31), GRABAR (79), PARNES (172), OUCHTERLONY (159, 160, 161), ISLIKER AND KLUTHE (100), KABAT AND MAYER (110) and CROWLE (48). Furthermore, an instructive and admirably complete handbook on the subject has recently been published by CROWLE (49). The present review based on about twelve hundred papers published in this field during the period 1956 to 1960 has been accomplished in order to guide those who are seeking recent technical developments and/or the increased information obtained by the application of these methods to the analysis of various biological materials.

The concept concerning antigens and the homogeneity of so-called pure substances of biological origin from the physiochemical point of view has gradually changed during the last decade. The increased sensitivity of new analytical methods has made it possible to detect impurities even in small quantities in materials

which earlier were considered pure, in this respect the immunological techniques have been particularly helpful. The power of the antibody-producing tissues in animals to recognize even minute quantities of, or qualitative differences in, antigenic materials is often remarkable. Thus the combination of suitable antisera and serological methods with a high resolving power, for instance, immunodiffusion methods, often provides excellent opportunities not only to study the composition of biological products but also to analyze qualitatively as well as quantitatively highly purified materials thereof for impurities. It has been stressed, however, that a limitation of the immunological methods used alone is that the analysis demonstrates only the existence and qualities of determinant groups and offers little or no information concerning the physiochemical character of the reacting antigenic molecules, *e.g.* their size, shape and structure. Therefore, techniques which comprise different types of analysis, *e.g.* immunodiffusion and immunoelectrophoresis, eventually combined with selective staining, can be advantageous as they offer supplementary information. Particular problems arise when the results of the different methods, as to distinguishing qualities, do not correspond. For instance, molecules of various composition, size and shape may carry exactly the same or closely related determinant groups and thereby seem identical when judged from the results of certain immunological tests. Material considered pure from a physiochemical point of view might show impurities detected by an immunological analysis revealing the existence of different determinants carried by more or less similar but not identical particles. It should also be considered that antigenic molecules may carry determinant groups in exposed, *i.e.* reactive, and or hidden positions. A further possibility which has to be taken into consideration is that similar molecules might carry determinants of more than one kind and in various combinations. It is feasible to assume that antigenic material of biological origin does represent the latter case even after fractionation by physiochemical and biochemical methods. Experimental results indicating the validity of this concept will be discussed later in this survey. For the moment it shall only be pointed out that the combination of physiochemical, immunological and biological analyses is essential for a satisfactory characterization of antigenic materials and the determination of the degree of their purity or homogeneity.

Gels for Immunodiffusion

The most commonly used stabilizing agent for immunodiffusion

analyses is agar. In addition other diffusion media have been tried (pectin, alginates and acetate cellulose).

Pectin

Pectin was introduced in 1956 by GRABAR, NOWINSKI AND GENERAUX (85) and among its advantages was mentioned the compartively low concentration (0.2–0.3%) necessary for the formation of a gel suitable for immunodiffusion analysis. Furthermore, there was mentioned the more easily controlled gelification at room temperature by enzymatic activity–pectin-methyl-esterase –and the possibility of easy elution by means of enzymatic digestion. On the other hand GRABAR (82) has later stated that the use of this type of gel might not be advisable due to, *e.g.* the instability of the enzymes to be used and the necessity of adding calcium ions for the gelification.

Alginate

The alginate method has recently been described by MORITZ (142). He recommends the use of sodium alginate – Protanal HF or SF of the Protan AB, Drammen, Norway – gelified under the influence of calcium sulphate. For the retransformation of the gel into a sol the use of a solution of the disodium salt of ethylene-tetra acetic acid is suggested. This solution does not dissolve eventual immune precipitates formed in the gel. The greater transparency of the alginate gel as compared to the agar is stressed and considered essential for the micronepholometric analysis of precipitates.

Cellulose Acetate Membranes

In 1959 CONSDEN AND KOHN (39) introduced cellulose acetate membranes as a medium for immunodiffusion. The dry membranes are commercially available (OXO Ltd.). When used for immunodiffusion they are soaked in the desired buffer solution, blotted and reactants applied as spots with capillary tubes. The membrane is then protected against evaporation by immersion in a bath of liquid paraffin and the diffusion takes place in the moist membrane. After washing in ether and afterwards in saline the membrane is stained with, for example, dilute nigrosine in order to make the precipitates visible. The membrane is said to allow "free diffusion" even of larger molecules, for instance, serum proteins, without adsorption. GRABAR (82), however, advises some restriction of their regular use for diffusion analyses as a filtering

effect on large or asymmetric molecules can be assumed. An obvious disadvantage of the method is that the formation of immunoprecipitates may not be followed during the analysis.

Agar

As has already been mentioned, however, the most commonly used medium for immunodiffusion is the agar gel. The character of this physiochemically poorly defined gelifying substance has lately been reviewed by *e.g.* GRABAR (82) and WIEME (213). Various brands of agar have been tested by different workers and considered more or less satisfactory for immunodiffusion. Difco Bacto Agar, Difco Special Agar (Noble), Oxo Ion Agar, Behringwerke Rein Agar and New Zealand Davis Agar are the products most often recommended. Even cruder products such as agar of Mexican, Korean or New Zealand origin have successfully been employed. Depending on the purity of the commercial product the agar may be utilized without further purification, however, each batch should be subjected to a diffusion test by a standardized immunoprecipitating system before general use. When a highly purified agar is needed, it may be obtained by means of various more or less elaborated methods, *e.g.* freezing and thawing, precipitation by ethanol, washing with ether, addition of charcoal and centrifugation or electrodialysis, COOPER (40), CROWLE (46), GRABAR (82), WIEME (213). A product with a nitrogen content not exceeding 0.015–0.03% may thus be obtained. It may be mentioned here that purification methods which include repeated melting should if possible be avoided due to the deleterious effect on the gelification process.

It is generally assumed that properly prepared agar gel with an agar content of about 0.3–1.5% does not interfere with a free diffusion of immunological reactants, antibodies and antigens of a moderate molecular size and a regular shape, see, for instance, ALLISON AND HUMPHREY (9) and VAN OSS AND HECK (150). The pore diameter of, for example, a 2% agar gel has been estimated at about 3 mμ. A simple method for analyzing the retarding effect of higher gel concentrations has recently been published by ALLISON AND HUMPHREY (8) –see Fig. 1. A different method applying a gel wedge arrangement has been devised by WUNDERLY (222, 223, 224). It should also be mentioned here that the agar gel may be of limited value for immunodiffusion analyses due to the retention or non-specific precipitation of a reactant by the agar, *e.g.* native fibrinogen, SELIGMANN, GOUDEMAND, JANIN,

BERNARD AND GRABAR (198), Lysozyme, KANINSKI (112), or serum β-lipoprotein, WIEME (213).

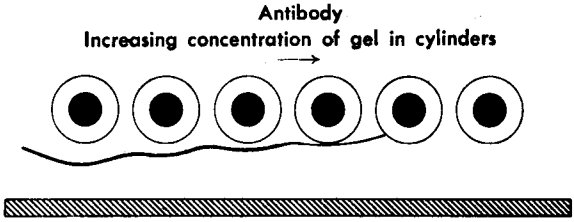

Fig. 1. Precipitation agar plate for demonstrating the retarding effect on diffusion of varying gel concentrations (Allison and Humphrey). Black, antigen; white, gel cylinders; shaded, antibody. Note the gradual dislocation of the precipitation line due to the retardation of the rate of diffusion caused by the increased concentration of the gel.

In order to prevent the growth of contaminating micro-organisms in the gel or on its surface an addition to the agar of sodium azide (0.01–0.05%) or merthiolate (0.01–0.02%) or phenol (0.1%) has been recommended. LE BOUVIER (30) has, however, reported "a deleterious effect" on precipitate formation by merthiolate. He also tried the addition of penicillin, streptomycin and mycostatin and reports that less defined precipitation bands were formed with these antibiotics present in the gel.

The addition of electrolytes to the gel used for immunodiffusion seems to be the regular procedure. Generally a content of 0.85% NaCl is used, but when chicken immune sera are analyzed 10% NaCl has been recommended, GRABAR (82). Various buffer solutions, *e.g.* a veronal buffer of pH 7.4, have also been employed. An enhancement effect of certain ions, Cd, Ni, La and Ce on the formation of precipitation bands has been investigated by CROWLE (45, 46). However, this enhancement is suppressed when the salt is mixed with the antiserum, thus it should be added to the antigen, or the gel containing the precipitate should be soaked in the salt solution. SILVERSTEIN, FEINBERG AND FLAX (199) have reported on a peculiar change of the transparency of the agar induced by antigen-antibody reactions. In diffusion plates they observed so-called "negative precipitin" lines, which manifested themselves as clear, thin bands transversing the somewhat cloudy agar. The immunosystem used was egg albumin, horse anti-egg albumin and the gel was hypotonic. A similar phenomenon was registered by GOUDIE, ANDERSON AND GRAY (78) when they tested human thyroglobin with a serum from a patient with Hashimoto's disease employing the agar diffusion plate technique.

Rabbit Cornea

It may be added that also a living transparent tissue – the avascular cornea of the rabbit – has been successfully employed as medium for immunodiffusion, GERMUTH, MAUMENEE, POLLACK, SENTERFIT, PRATT-JOHNSON AND VAN ARNAM (67).

REGISTRATION OF IMMUNOPRECIPITATES

Densitometers

For the registration of site, shape and intensity of precipitation bands or lines in agar plates or tubes microdensitometric measurements sometimes have been performed. My own experience comprises only the use of two instruments, a recording photoelectric microphotometer – Schnell Photometer II, Zeiss, Jena–, OUCHTERLONY (158) and a recording microphotometer – Jarell-Ash Company, Boston, Mass. Both instruments were originally designed for the reading of spectrograms but can easily be adapted to direct recording of precipitates in plates or tubes. The former instrument is suited for transmitted light measurements, the latter for scattered light measurements as well. The instruments can also be utilized for densitometric analysis of photographic plates recording precipitation spectra. HAYDEN AND BECKER (92, 93) have employed another type of densitometer – Bausch and Lomb, Rochester, N.Y. A turbidimeter (Libby Photronreflectometer) specially redesigned to record precipitation bands in agar columns in narrow tubes has been reported on by GLENN – instrument SAMA (69), SAMI (70) and SASI (71, 72, 74, 75). The instrument is particularly recommended for quantitative analyses, when great accuracy is needed and a rapid recording is desired.

Photographic Methods

For the registration of precipitation patterns in gels a variety of photographic methods have been used as reported in the earlier survey, OUCHTERLONY (158) – see Fig. 2. Some modifications of the photographic procedure have later been reported. For the scattered light illumination of plates, beams of light as nearly parallel to the surface of the agar as possible have been suggested, *e.g.* strong spotlights, HUNTER (99), movable double annular fluorescent tubes, SCHUTZ (196), or a central Photo-flood bulb with a reflector set of flat mirrors and a conical reflector, RUDGE (188). A ribbon-filament microscope lamp combined with a substage mirror for the reflection of the beam at a suitable angle has also

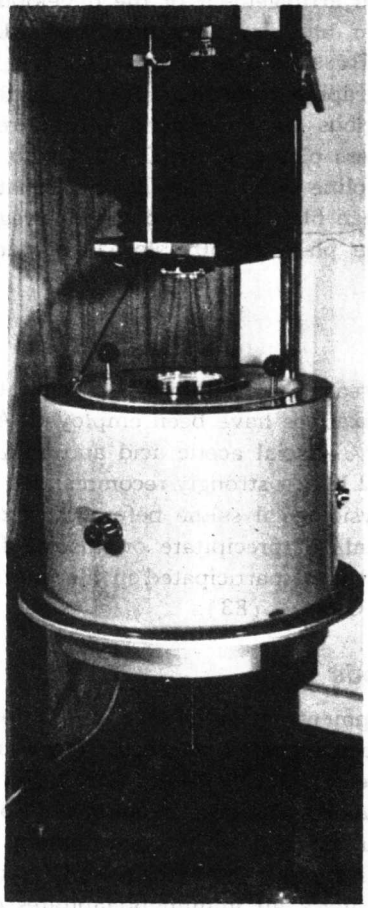

Fig. 2. Apparatus for photographic registration of precipitation patterns in gel. The circular fluorescent light source is enclosed in such a manner that scattered light illuminates the plate.

been used, MURCHIO (143). An annular source of light has been described by REED (181). For transmitted light registration of precipitation plates the use of a photographic enlarger has been recommended, DIKE (51). The plate lowered into glycerol is substituted for the negative in an adapted negative holder close to the condenser and the image projected on photosensitive material. A method for making a direct photogram on a lantern plate of the precipitation plate has been recommended as saving in time and not involving the use of special photographic equipment, JONES AND MARSHALL (109). Instead of lantern plates contrasty bromide printing paper may be utilized, GRABAR (83). A chrono-photo-

graphic registration arrangement of the development of immunoprecipitates in agar has also been described, MANIGAULT (133) and MANIGAULT, BUSSARD AND OUDIN (134).

For the photographic recording of precipitating patterns film and plates of various trademarks have been recommended, e.g. Ilford N. 40 Process plates, Ilford G 50 Ortho halftone backed plates, Ilford Ortholine G 5.51 film, Kodak Panatomic X film and Contrast process pan film. My own choice for regular use is Ilford Thin film halftone plate N. 50 (slow, blue sensitive, high contrast).

Fixatives

In order to increase the visibility of precipitates formed in the agar gel various fixatives have been employed. A satisfactory one is a mixture of 6% glacial acetic acid and 94% saturated picric acid solution, pH 2.1. It is strongly recommended that the gel plate be washed in physiological saline before the fixation in order to avoid large amounts of precipitate originating from those serum proteins which have not participated in the immune reactions, e.g. BERGRAHM (27), GRABAR (83).

Staining Methods

Further developments of staining methods for immunoprecipitates have also been reported. A recent review of the experiences in this field has been given by URIEL (205). For the non-selective staining of immunoprecipitates Bromphenol blue, Azocarmine, Nigrosin, Ponceau red and Light green, among other dyes, have been utilized. It is stated that water soluble stains are preferable. These stains will mark all immunoprecipitates due to their antibody-protein content. Selective staining methods have also been employed, e.g. Oil red or Sudan IV for lipoids and lipoproteins, periodic acid – Schiff's or Nadi's reagent – for polysaccharides or glycoproteins, Feulgen's fuchsin for DNA, Alizarin blue S for copper containing proteins. The experience of our laboratory, however, is that it is often difficult to obtain clear-cut results regarding the composition of precipitates, when selective staining methods are used – see also RONDLE AND CARMAN (186). For the general staining precipitation spectra for a permanent record we prefer Light green. SCHEIDEGGER (192) and FRANCQ, EYQUEM AND GRABAR (62) have stated that fluorescent precipitation lines may be obtained when labelled antibodies – sulpho-fluorescin or sulpho-rhodamin – are employed for immunodiffusion analyses. Isotope labelled reactants have also been employed in order to facilitate

a selective registration of precipitation lines or bands, ALLISON AND HUMPHREY (9), ALY AND GILLICH (10), KEUTEL (115), SALMON (189) and REJNEK AND BEDNARIK (182). It may also be mentioned that for structural analyses of precipitation bands the electron microscope has been successfully utilized, EASTY AND MERCER (52).

TECHNIQUES OF SIMPLE DIFFUSION

Simple One-dimensional Diffusion

Mathematical Theories

The further elaboration of the simple one-dimensional diffusion method in tubes worked out by OUDIN has mostly been employed to analyze factors influencing the formation of precipitation bands and the displacement of these bands. OUDIN'S (164) three original formulae for quantitation of antigen and antibody are as follows:

$$1)\ \frac{h}{\sqrt{t}} = k \quad 2)\ \frac{h}{\sqrt{t}} = \gamma \log \frac{Ag}{Ag_0} \quad \text{and} \quad 3)\ \frac{h}{\sqrt{t}} = \alpha \log \frac{Ab}{Ab_0}$$

In these formulae h is the displacement of the leading edge of the precipitate in the agar column after a time t, γ and α are constants and Ag and Ab the initial concentrations of antigen and antibody, Ag_0 and Ab_0 the corresponding extrapolated values for $\frac{h}{\sqrt{t}} = 0$. The formula no. 2 refers to conditions where the concentration of Ab is constant and that of Ag variable and the formula no. 3 applies to the reverse conditions. OUDIN (166) and BECKER, MUNOZ, LAPRESLE AND LE BEAU (23) have further stated and experimentally verified that there is a linear relationship between k and the logarithm of the concentration of the antigen for low values of $h/\sqrt{t}$, and between k^2 and the same logarithmic values for high values of $h/\sqrt{t}$. BECKER et al, have also for the last case pointed out the relationship between the slope of the curve and the diffusion coefficient of the antigen if MITCHISON AND SPICER'S (139) equation for agar tube diffusion is applied. These statements, based on calculations where various diffusion equations have been applied and several assumptions concerning the experimental conditions have been postulated, have been supported by numerous investigators but have been critically discussed by others, *e.g.* AUGUSTIN (13), AUGUSTINE AND HAYWARD (16), SPIERS AND AUGUSTIN (200) and AUGUSTIN, HAYWARD AND SPIERS (17).

They have presented and experimentally tested a different mathematical theory concerning precipitation band formation in OUDIN tubes and its effect on quantitative analysis and determination of diffusion coefficients of the reactants. The elaboration of their formulae takes such factors into consideration as changes of the antigen concentration at the liquid/gel interface, disturbances in the free diffusion due to the precipitation, the formation of soluble Ag-Ab complexes, the variation of R – antigen/antibody ratio – at different sites in the gel column, the influence of the finite length of the agar column, etc. The applicability of their calculations particularly as compared to the deductions made in BECKER'S "elementary theory" is given in their two papers (13, 17), containing a considerable amount of experimental data. Their equation predicting the variation of $h/\sqrt{t}$ in relation to varying ratios of concentrations of Ag and Ab is as follows, and should be compared to, see OUDIN'S (164, 165) previously published diagrammatic presentation of the same relationships:

$$\frac{[1+\mathrm{erf}(k/2\sqrt{D_1})]\exp(-k^2/4D_2)]}{[1-\mathrm{erf}(k/2\sqrt{D_2})]\exp(-k^2/4D_2)]} = \frac{C_1}{C_2}\sqrt{\frac{D_1}{D_2}}$$

D_1 and D_2 are the diffusion coefficients and C_1 and C_2 the initial concentrations – in equivalent units – of the Ag and Ab respectively, exp stands for the exponential function and erf is the normal probability integral.

NEFF AND BECKER (147) and BECKER AND NEFF (24, 25, 26) have further elaborated their original "elementary theory" and have presented a series of publications taking into account the diffusion as well as the interaction of antigen and antibody. They have applied an equation deduced by WALES.

$$\frac{Ag}{Ab\,R'} = \frac{z\,(1+\mathrm{erf}\,z)\,e^{z^2}}{y\,(1'+\mathrm{erf}\,y)\,e^{y^2}} \quad \text{where} \quad y = \frac{k}{2\sqrt{D_2}} \quad \text{and} \quad z = \frac{k}{2\sqrt{D_1}}$$

Ag and Ab represent the concentrations of antigen and antibody respectively, R' is the ratio of antigen to antibody at the leading edge of the precipitation band, erf is the normal probability integral and D_1 and D_2 the diffusion coefficients of antigen and antibody respectively. Experimental evidence is presented showing that this equation holds over a wide range of antigen and antibody concentrations. For the estimation of antibody concentrations BECKER AND NEFF have by an approximation derived the following equation using the procedure of MITCHISON AND SPICER (139):

$$k=4.08 \frac{\sqrt{D_1 \cdot D_2}}{\sqrt{D_1}+\sqrt{D_2}} \left[(\log Ag - \log \frac{R'}{r} Ab) \right] \quad \text{where } r = \sqrt{\frac{D_1}{D_2}} \text{ and}$$

$R' = r R_0$. R_0 denotes the ratio of antigen to antibody at zero movement of the precipitate, *i.e.* $k = 0$ .The validity of this equation is tested experimentally by BECKER AND NEFF (26) and the results should be compared to those obtained by AUGUSTIN (13) and AUGUSTIN et al. (17).

Influence of Specific and Nonspecific Substances on the Analytic Results

It is interesting to note that the difference in opinion between the former and latter groups of investigators has stimulted an interest in investigations of various factors, specific as well as nonspecific substances, which affect the analytic results obtained with the OUDIN tube method. Thus OUDIN (167) for instance, has reported on the changes of the value of k due to the presence of substances other than antigen and antibody in the external reagent layer. Such substances were denoted n. s. s. (nonspecific substances) and sodium cacodylate, chloride or phosphate, glucose, polyvinylpyrrolidone, mammalian sera and snail hemolymph were given as examples thereof. RUBINSTEIN (187) has reported on misleadingly high values of k due to increased saline or protein content of the medium in which the antigen was suspended – a gradual influence, however, reaching a maximum at a 2–2.5% saline or protein concentration. PREER AND TELFER (180) demonstrated that such disturbances may be explained by the gravity-induced convection currents in the liquid antigen layer. Thus density gradients of n. s. s. established at the liquid/gel interface might cause variations in the k value, an error which can be avoided if the external reactant layer is also gelified. The influence on the k value of another factor – viscosity – was also investigated. Following an original observation by NEFF AND BECKER (146) concerning this factor PREER AND TELFER (180) found that differences within certain limits viscosity of the antigen layer did not detectably influence the k value. An increase of the viscosity of the antibody layer due to the addition of normal serum had an influence which could be predicted from the fact that the diffusion coefficient is inversely proportional to viscosity, therefore k is inversely proportional to the square root of viscosity. On the other hand when sucrose was used to increase the viscosity less clear-cut results were obtained, which indicated a third mechanism influencing k in addition to convention currents in the antigen layer and