Handbook of Experimental Immunology

Third Edition

Edited by **D.M. Weir**

Volume 3

Application of Immunological Methods

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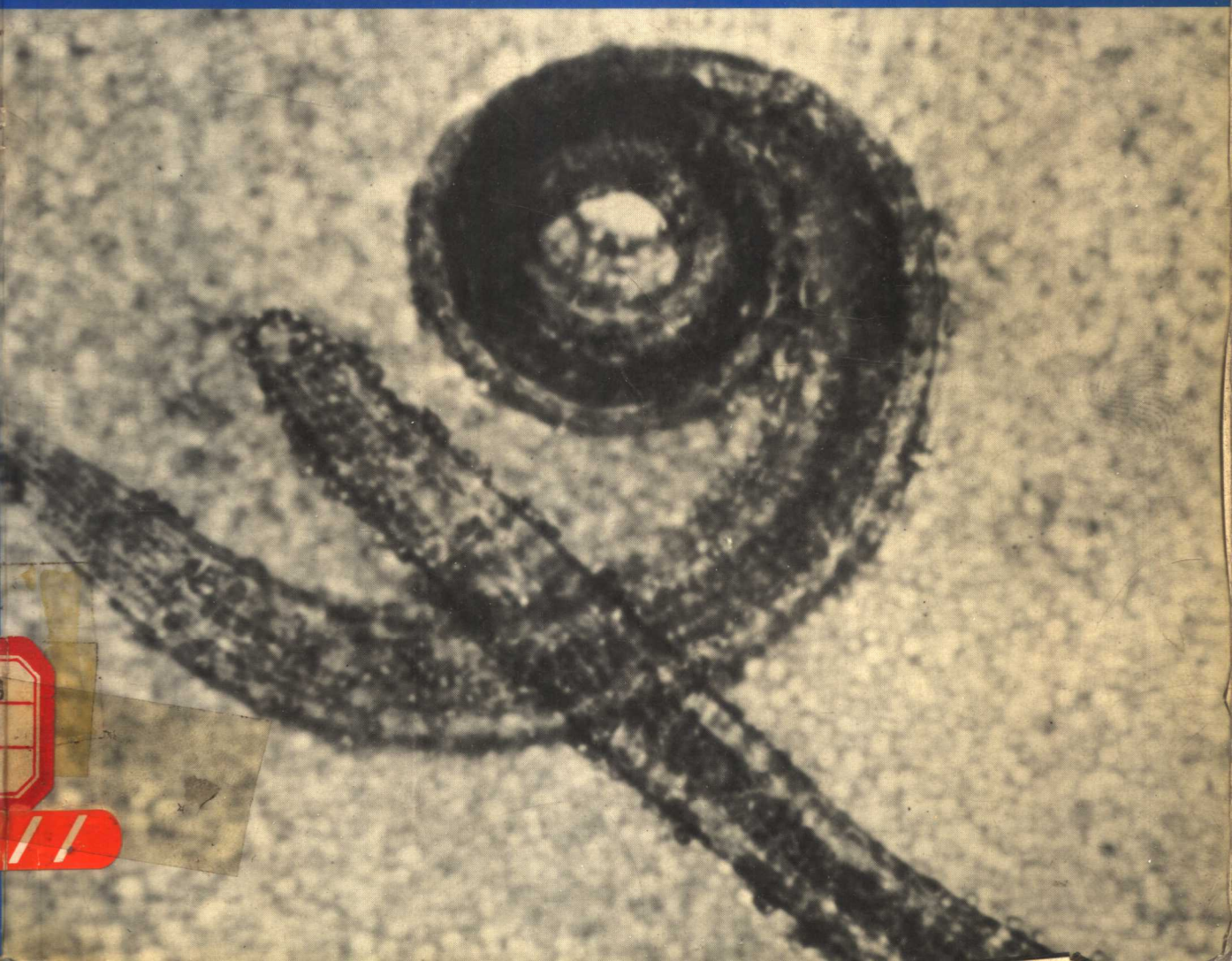

HANDBOOK OF EXPERIMENTAL IMMUNOLOGY
IN THREE VOLUMES

Volume 3
Application of Immunological Methods

EDITED BY

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THIRD EDITION

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Preface

Experimental immunology has continued to grow exponentially since the last edition of this text and this is reflected in the new material and increased number of contributors to this new edition. Like the previous edition the material in the 3rd Edition has been grouped into the three main subdivisions of experimental immunology. The chapters in each subdivision have been brought together into sections Thus in Volume 1, Immunochemistry, there are sections on antigens, complement, immunoglobulin purification and characterization, immunogenetics and antigen–antibody interactions. In Volume 2, Cellular Immunology, under the heading of lymphocytes, there are chapters on a large variety of procedures for the preparation, separation, analysis, preparation of hybrids and demonstration of activities of lymphocytes. Similarly there is a section on phagocytes and a section on cellular antigens and products.

Volume 3, Application of Immunological Methods, contains chapters on immunological methods in bacteriology, virology, mycology, protozoology and helminthology. There are chapters on mediators and antibodies in hypersensitivity reactions and a chapter on tests of immune function. The appendices contain discussion of statistical aspects of planning and design of immunological experiments statistical analysis of data, adjuvants and immunization, laboratory animal techniques and mouse-breeding procedures.

There has been extensive revision of the majority of chapters and some of the material from earlier editions has been deleted. There are many new chapters on methods that have come into use since the last edition.

This book is directed primarily at research workers in each of the three subdivisions of immunology which cover a wide range of scientific disciplines. Workers in the field tend to specialize within a limited area, so that the availability in this text of discussion and description of methodology covering each of the main subdivisions should be particularly valuable. Techniques outside the normal expertise of most experimental immunologists are thus made accessible. The inclusion of appendices on experimental design and statistical analysis of immunological data should also be convenient and useful aids in the planning of experiments and analysis of data.

I should like to thank the many contributors for their efforts and co-operation that have made it possible to bring together information on a very wide range of immunological topics and for their help in trying to fulfil the requirements for a uniform format for the presentation of the material.

Thanks are due to the many colleagues who have offered constructive advice arising from the use of earlier editions. Dr Len Herzenberg has encouraged us to prepare supplements to the text as important new procedures are described. It should thus be possible for the reader to gain access to new material within a short time of its original description without having to wait for a new edition of the complete text. Contributors and readers are encouraged to inform the editor of interesting new developments in their field.

The index was prepared by Mr S. Revett and the sub-editing carried out by Linda Adler and Rosemary Walton. Dr Caroline Blackwell provided editorial advice on microbiological aspects of the text. I should like to acknowledge the invaluable and highly efficient support provided by the publishers, particularly Mr Per Saugman and Mr Nigel Palmer and his staff of the Edinburgh office.

D. M. Weir

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This section will be concerned primarily with immunological techniques used in bacteriology related to human and veterinary studies. During the past few years there has been a growing realization of the limitations of traditional serological techniques in bacteriology. Problems of interpretation have arisen when newly-discovered, and not fully understood, immunological phenomena have been utilized as the basis of tests in clinical, epidemiological and experimental situations, and there has been much needed attention given, in the last few years, to their relevance to the clinical situation. The application of test procedures for the localization and visualization of staphylococcal protein-A using immunoperoxidase and electron microscopic procedures, is likely to be deferred until further assessment is made of their clinical relevance. The clinical relevance of other procedures, however, e.g. the solid-state and ELISA techniques, have been rapidly established. This chapter has therefore been written with a view to presenting some guidance to the selection of immunological methods that are available for experimental and clinical investigation. Some techniques are given in considerable detail. Others are only briefly summarized. The complexity and variation of antigens which may be used in tests are inevitably immense, and for results to remain comparable the method of antigen preparation will require meticulous care. The importance of strain selection and cultural techniques cannot be overstated.

Agglutination

Though clinical reliance is placed upon standard direct agglutination methods in studies of *Brucella*, *Leptospira*, *Francisella*, *Yersinia* and *Bordetella*, other agglutination phenomena are sometimes used to increase sensitivity and specificity. Co-agglutination [19] can be comparable in accuracy with counter-current immunoelectrophoresis and it has been widely applied to the investigation of *Streptococcus* Lancefield groups. *Listeria* and *Kurthia* may also be grouped using co-agglutination with *Staphylococcus* protein A.

Co-agglutination in streptococcal grouping

Co-agglutination is performed by mixing test bacteria with antibody-coated *Staphylococcus* preparations.

Method

Streptococci are grown on 5 per cent blood agar and emulsified in Tris buffer 0·03 mol/l pH 8·1 and trypsinized for 1 hour at 37° C at a final trypsin concentration of 1 mg per ml. Cowan I strain of *Staphylococcus aureus* NCTC 8530 is grown in trypticase-soy broth (Kelsey-Marshall nutrient broth is not advised, owing to some inconsistency of subsequent co-agglutination) and cells washed in phosphate buffered saline (PBS) pH 7·3. After adequate formaldehyde treatment and repeated washing in PBS, the suspension is kept at 80° C for 30 min and adjusted to 10 per cent in PBS. The cells are added to specific antiserum (10:1 vols) at 37° C for 1 hour, washed and resuspended to 1 per cent. Equal volumes of the two organism preparations are mixed on glass slides and examined after agitation for 30 seconds [19]. Absorption of commercially available serum may be required [62]. Should commercial antisera be employed, Difco preparations are recommended.

Haemagglutination

Haemagglutination (HA) using *Treponema pallidum* antigen-coated red cells has been found to be technically simpler and more specific than immobilization tests [41, 54] and HA efficacy appears to match that of fluorescein-antibody procedures [28] (see Chapter 20). The manual procedures of a decade ago [53] have rapidly given way in the routine laboratory to automated procedures [49]. Leptospira antigen-sensitized, human group O erythrocytes fixed with glutaraldehyde [4] or pyruvic aldehyde are reliably genus-specific in the early stages of disease. This method is effective in detecting the rapid convalescent antibody decline following self-limiting experimental animal infection.

The indirect haemagglutination test (IHA) in *Pseudomonas pseudomallei* infection [1] detects antibody responses within 7 days of disease onset. Transient seronegatives are so far unexplained. The low titres to *P. pseudomallei* observed in treponemal, mycobacterial, rickettsial, leptospiral and some viral infections have been only superficially investigated. The comparable titres detected in *Actinobacillus mallei*, *P. stutzeri* and *P. aeruginosa* infections depend on shared antigens.

Passive HA is well documented over many years as having diagnostic value [27] in *Corynebacterium diphtheriae* infections using formalinized, tanned sheep erythrocytes, and employing toxin with or without bis-diazobenzidine to facilitate adsorption [13]. Adequate correlation has been demonstrated between PHA and the rabbit-skin method of toxin neutralization [47]. Safety considerations in experimentation with *Francisella tularensis* have caused reliance to be placed on HA [18]. The use of antigens derived from avirulent *F. tularensis* strain ATCC 6223/B38 is traditional, but antigens from virulent *F. tularensis* strains give a more sensitive HA test. The HA test in experimental *Listeria monocytogenes* infections has been superseded, though its status in the study of human listeriosis and shigellosis is unjustifiably low [14]. For recognition of specific antibody in natural and experimental *Yersinia enterocolitica* infections the problem of common enterobacterial antigens may be minimized by inhibition and absorption procedures [50]. Using purified *Vibrio cholerae* toxin, HA shows good correlation with the skin permeability factor neutralization test. The sheep erythrocytes used in this method are stable in dilute thimerosal for at least 6 months [35]. The antibody response to *Neisseria meningitidis* outer cell membrane antigens in natural infections with the organism is specific for protein, lipopolysaccharide and polysaccharide components from group B and C strains of many serotypes and haemagglutination tests using such antigens are valuable [67]. The differential elevations of titres to these antigen classes among nasopharyngeal carriers and non-carriers suggest more selective ways of investigating the specific human immune responses to *N. meningitidis* than immunofluorescence [2]. However, HA is of lower specificity than neissericidal-antibody tests. The IHA and haemagglutination inhibition test (HI) may be employed to elucidate several serological specificities within LPS obtained from members of the *Bacteriodaceae* [36]. Analogy with the O antigens of the *Enterobacteriaceae* is suggested by the susceptibility to periodate oxidation.

HA using sheep erythrocytes sensitized by rabbit anti-sheep red blood cell (RBC) gammaglobulin and subsequently exposed to streptococci, is unaffected by pepsin and trypsin treatment though abolished by *Streptomyces griseus* protease. Only intact IgG

appears to be involved; neither $F(ab)_2$ or Fc from IgG, nor IgA, IgM, IgD, or IgE react by this method [20]. Different *Streptococcus* isolates exhibit varying abilities to agglutinate the coated red cells, which may range from all (group G) to only one-third (group D). Heat treatment does not affect the phenomenon, which promises advantages in bacteriological grouping procedures and investigative taxonomy. Serological appraisal of group A streptococcal infections by antistreptolysin O and anti-DNase-B serum assay may be improved 50 per cent by the use of HA, employing in this case human group O erythrocytes sensitized by group polysaccharide esterified with myristoylchloride [29]. Direct 'cold' haemagglutination of 1 per cent human O erythrocytes is useful in the investigation of *Mycoplasma pneumoniae* infections when used in conjunction with the traditional complement fixation test (CFT) [57]. The IHA test when used alone in human T-mycoplasma infections is less sensitive than metabolic-inhibition tests [8], but is more sensitive than direct and indirect immunofluorescence and growth-inhibition techniques [7].

HA, *Yersinia pestis* antibody

Of the several HA methods available the following slightly modified technique from Rust *et al.* is selected for description. It relies on the use of Baker's crystalline *Y. pestis* antigen [3] attached to tanned aldehyde-treated erythrocytes [48]. Preliminary titration-standardization of each preparation minimizes aberrances due to the molecular heterogeneity of the Fraction One (F-I) antigen and non-specific agglutinations due to antigenic contaminants [64].

Antigen preparation

Stock lots of F-I are extracted from designated dried, acetone-killed *Y. pestis* strains and stored at −20° C in concentrations of 2·5 mg per ml in Sörenson's phosphate buffer, 0·067 mol/l, pH 6·0. Sheep erythrocytes collected in Alsever's solution are washed in saline and adjusted to a 50 per cent suspension. Volumes in ratios 1·5:7·0:0·7 of 25 per cent pyruvic aldehyde, 1 per cent sodium carbonate and Sörenson's 0·067 mol/l phosphate buffer at pH 8·0 are mixed and one volume sheep erythrocytes stirred in. After 18 hours at 4° C the pyruvic-aldehyde treated erythrocytes are separated by low-speed centrifugation and washed in 1 per cent absorbed, inactivated rabbit or mouse serum-saline. The erythrocytes are exposed to 0·05 per cent tannic acid in the Sörenson buffer pH 6·0 (1:8 vols) and incubated for 1 hour at 26° C before serum-saline washes. The sensitized pyruvic-aldehyde tanned erythrocytes may be stored in 0·1 per cent sodium-azide–serum-saline at 4° C for up to 4 months. It is imperative to wash the stored tanned erythrocyte preparations in fresh serum-saline before use. Best results with lyophilized preparations are obtained by reconstitution in serum-saline for 2 days followed by two washes after a further 24-hour interval.

Titration

Sodium azide can be employed. Cells are used as 0·5 per cent suspensions. Controls include tanned, sensitized and non-sensitized sheep erythrocytes, and human antisera against non-F-I protein antigen as a specificity monitor. Test sera are absorbed and inactivated by adding to each serum one-tenth its volume of a 50 per cent suspension of non-sensitized pyruvic-aldehyde tanned sheep erythrocytes. These mixtures are then incubated at 24° C for 30 min, followed by 30 min at 56° C. Cells are afterwards centrifuged and microtitrations performed on serum dilutions made in serum-saline. Paired titration sets are incubated at 25° C for 24 hours before reading. Those dilutions at which haemagglutination is specific to the particular F-I antigen preparation used, producing no reaction with non-specific antiserum controls, represent the optimum antigen concentration for the test. Close correlation exists between results obtained by means of tests using pyruvic-aldehyde tanned sheep erythrocytes and orthodox standard HA tests [56].

Haemagglutination (HA) test using Streptococcal polysaccharide conjugates

The sensitivity and reproducibility of HA in the study of group A streptococcal polysaccharide (APS) can be increased by employing stabilized human erythrocytes coated with conjugates of APS and human gammaglobulin. This procedure lends itself to HA-inhibition studies, and to the investigation of specificity. It is also a sensitive means of selecting points of interspecies differential immunogenicity.

APS preparation and conjugation

Group specific polysaccharide from type 12 group A beta-haemolytic streptococci is assayed against L-rhamnose standards [34]. Conjugation of APS with human gammaglobulin (HGG) follows the system

used in coupling amino group ligands to agarose. The APS in 0·1 mol/l $NaHCO_3$ is stirred continuously at 18° C and the pH altered from 8·3 to 11·0 by the slow addition of 1·0 N.NaOH. A weight of cyanogen bromide equal to that of the APS is added in a small volume of distilled water, the pH being held at 11·0 by addition of normal NaOH. Sufficient 1·0 N.HCl is added at a temperature between 0 and 4° C to alter the reaction pH to 10·0. This is done immediately prior to addition of a weight of HGG equal to twice that of the APS employed. The mixture is adjusted to pH 9·0, stirred for a day at 4° C and dialyzed against 0·01 mol/l neutral PBS, after which the protein content of the conjugate (A-G) can be determined against bovine serum albumin standards.

Preparation of streptococcal antiserum
Group A beta-haemolytic *Streptococcus pyogenes*, cultured in glucose-salts supplemented Todd-Hewitt broth to the midpoint of the exponential growth phase, is inactivated by the addition of phenol to 1 per cent concentration. After washing in 0·01 mol/l PBS pH 7·0, 10 mg amounts in saline are emulsified in Freund's complete adjuvant, and antiserum produced in rabbits. A 1·5 ml volume of the vaccine per animal is distributed in various subcutaneous and intramuscular sites. The animals are bled after 2 weeks.

Preparation of double-aldehyde stabilized erythrocytes (DASE)
Pooled fresh human group O *cde/cde* erythrocytes are subjected to double-aldehyde stabilization at 24° C in 0·11 mol/l phosphate buffer at pH 7·2. Equal volumes of 3 per cent pyruvic aldehyde and 8 per cent erythrocyte suspension are stirred for 18 hours and the mixture subjected to a single coarse-gauze filtration before repeated washes in buffer. The packed-cell volume of the pyruvic-aldehyde treated erythrocytes is restored to 8 per cent, and the above process repeated using a 3 per cent formaldehyde-buffer solution. The DASE preparations are stable for 6 months at 4° C as a 10 per cent suspension in the same buffer.

Coating of DASE
The A-G conjugate or human gammaglobulin (as antigen) is added to 1 per cent DASE suspension in 0·1 mol/l acetate buffer pH 4·0 and stirred at 24° C for a duration determined by the antigen used. The coated DASE are then washed in 0·11 mol/l phosphate buffer pH 7·2. The optimum conditions for the adsorption of A-G as determined by Hirata *et al* were 10 μg A-G per ml DASE suspension for 30 min, and 10 μg HGG per ml DASE suspension for 10 min. Slightly longer periods than these, however, may be used.

The haemagglutination procedure for coated DASE preparations is carried out using 0·1 per cent gelatin in 0·11 mol/l phosphate buffer pH 7·2 as the serum diluent. Standard microtitration volumes and procedures are then followed, with the DASE suspension adjusted to 0·25 per cent in buffer and establishment of appropriate controls. Brisk shaking for 30 seconds is recommended before incubation for 1 day at 24° C [34].

Modified antiglobulin-consumption test

The antiglobulin-consumption test, as originally applied to recognition of autoantibodies in tissues, relied upon the reduction in titre of anti-human globulin (AHG) following its overlay on tissue cells. Though AHG ('Coombs') reagents are in common use in bacteriology the adaptation of antiglobulin-consumption tests to the preliminary selection of sera containing antibacterial antibodies has only recently been carried out. Hinchliffe's modification aims at the recognition of brucellosis by a defined consumption test.

Method [33]
Weybridge standardized *Brucella abortus* preparations are employed and washed several times prior to use. Commercial AHG reagents can be used with sensitized red cells. Equal volumes of antigen (dilution 1:10) and test serum dilution are incubated 15 min at 37° C, centrifuged and the pellet resuspended in twice the original volume of diluent. After repeated washings, AHG appropriately diluted is added, the organisms are resuspended and finally centrifuged after the usual Coombs-test incubation procedure. Sensitized erythrocytes are now added to the separated supernatants and the plates read for agglutination after 30 min at room temperature.

Results by the conventional Coombs AHG test, mercaptoethanol agglutination and complement fixation are comparable to the antiglobulin-consumption test, the sensitivity of which may be increased slightly by employing antigen dilution at 1:5. No benefit is conferred by longer incubation.

Immunofluorescence (IF) (see Chapter 15)

Fluorescent antibody techniques are used in bacteriological investigations in direct, indirect or two-stage, and in complement-using or three-stage procedures. The methods have contributed to the investigation of *Neisseriaceae*, in studies of respiratory pathogens and in studies of *Streptococcus*, *Shigella*, *Corynbacteria*, *Yersinia*, *Francisella*, *Listeria* and *Bacillus anthracis*. Cross-absorption studies using unconjugated antisera prepared against endotoxins have demonstrated common antigens among the *Enterobacteriaceae*, particularly *Aeromonas*, *Escherichia* and *Shigella* [45].

Microimmunofluorescence in *Chlamydia*

Microimmunofluorescence techniques have superseded the radioimmune precipitation technique, which is, like complement fixation in human *Chlamydia* infections such as 'non-gonococcal urethritis', largely a group-reactive phenomenon. Microimmunofluorescence enables serotyping studies to be performed and provides reliable epidemiological data for chlamydial strains [55], particularly in trachoma-inclusion conjunctivitis and lymphogranuloma venereum. Simplified immunologically based classification procedures, using monotypic mouse antiserum to fresh isolates and employing microimmunofluorescence, have resulted in the identification of new strains [46]. Experimental immunization studies in the mouse and chimpanzee may be monitored serologically by microimmunofluorescence, as can conjunctival antibody levels in infected animals.

Method

Infected yolk sacs showing a high density of *Chlamydia* elementary bodies are crushed and suspended to 2·5 per cent in 0·01 mol/l PBS pH 7·0 for storage at $-65°$ C. Reference antisera prepared in mice are used to check antigen batches, which are then dotted onto large microscope slides, dried and then acetone-fixed. Doubling dilutions of serum in PBS pH 7·0 are made and cross-tested in grid pattern against the fixed antigen drops using the indirect IF technique, with commercial goat antisera to human IgM, IgG and IgA labelled with fluorescein isothiocyanate. Rhodamine-conjugated bovine albumin is the recommended counterstain.

'Defined' immunofluorescence techniques [17] employing FITC-labelled $F(ab')_2$ fragments of IgG are of use in identifying isolates of selected groups of pathogens within problem genera, such as the *Streptococcus*.

Radioisotope-antigen binding assay, double antibody technique

The quantitative assay summarized here employs the double-antibody technique [12] derived from earlier insulin assays [51], and employs ^{125}I-labelled pili preparations from *Neisseria gonorrhoeae* type 2 (T2). The assay is applicable to human natural or experimental disease, to rabbit inoculation studies and to the comparison of vaccine preparations. *N. gonorrhoeae* T1 and T2 pilated strains correlate with infectivity in humans and the chick embryo models [11, 43].

Pili preparation

Pili are collected from cultures of *N. gonorrhoeae* T2 at the completion of the logarithmic growth phase in fluid medium. The pili are precipitated by addition of sufficient HCl to alter the supernatant's pH to 4·0. The precipitate is extracted and redissolved in 0·01 mol/l Tris buffer pH 7·5 with 0·01 mol/l NaN_3 at 4° C before centrifugation and overnight dialysis (4° C) against distilled water. Magnesium chloride (final concentration 0·1 mol/l) is then added. A further brief centrifugation at $12\,000 \times g$ is completed 24 hours later before a similar resuspension in Tris pH 8·0. The preparation is then dialysed over 3 days against large volumes of buffer. Centrifugation and subsequent dialysis against distilled water precede repeated $MgCl_2$ precipitation and dissolution in the Tris pH 8·0. The extent of purification may be judged by potassium phosphotungstate or uranyl acetate negative staining and electronmicroscopy and electrophoresis in 1 per cent polyacrylamide gels. For the latter estimation Buchanan *et al* [12] recommend 1 μg of ^{125}I-labelled pili preparation (by the chloramine T method, Chapter 14), or appropriate molecular-weight marker boiled briefly in 1 per cent SDS, 1 per cent 2-mercaptoethanol before application to the gel and subsequent radioestimation of 1 mm gel slices. The pili preparation is stored at 4° C in 0·01 mol/l Tris (pH 7·0) with 0·01 mol/l NaN_3.

Rabbit antiserum preparation

Adult New Zealand Red rabbits are inoculated with 50 μg of the pili material in complete Freund's

adjuvant (0·1 mg per ml *Mycobacterium butyricum*), this amount being distributed intramuscularly and subcutaneously (Appendix 4). A month later this is repeated. Rabbits are bled after a further 10 days, and the gammaglobulin fraction conjugated to fluorescein isothyocyanate (1:2·5 ratio) for tests of *N. gonorrhoeae* strain specificities and possible interspecies cross-reaction. Standardization of the antigen-binding assay is done by means of separated IgG dilutions obtained from rabbit antisera.

Antigen-binding assay

Azide is removed by dialysis against water, and 40 μg pili preparation labelled with ^{125}I (50 μg chloramine T; 200 μCi). Labelled pili of specific activity 2000 cpm/ng is prepared at a concentration of 0·5 mg per ml in 1 per cent bovine serum albumin made up in PBS pH 7·2. ^{22}Na may be used as a volume marker. Test serum is heat-inactivated and diluted to 1:31 in PBS pH 7·2 with 1 per cent bovine albumin and antigen added (2:1 vols), the mixture being kept at 4° C overnight. Twice the volume of goat anti-human or anti-rabbit immunoglobulin antiserum necessary to precipitate control ^{125}I-labelled immunoglobulin preparations is now added. The precipitate is centrifuged out and separately counted as ^{125}I and ^{22}Na to obtain the percentage antigen bound to antibody. Pili preparations made following this method represent approximately 1 pilus per cell, 10 per cent of the average [40]. This technique has the benefit that ^{125}I-labelled pili appear morphologically identical with unlabelled preparations. Further advantages of employing single protein subunit aggregates as antigen are the sensitivity achieved (100 pg of antibody), and the detection of increments in antibody against pili during the early stages of infection. The technique has also been used to demonstrate the raised titres among female asymptomatic carriers, and in the investigation of animal models.

Bactericidal antibody tests

Despite difficulties of technique, bactericidal antibody tests have been used extensively in the study of responses to infection with *Vibrio cholerae* [5], *Francisella tularensis* [61] and *Neisseria meningitidis* and *N. gonorrhoeae*. Antibody response in human *N. meningitidis* infection is closely related to immunity [30]. Owing to the considerable antigenic variation which occurs among *Neisseria* sub-groups this correlation can be detected more effectively by the use of autogenous strains in the assays. Limiting bactericidal antibody tests to one serum dilution, or to a narrow range of dilutions, in the form of 'screening' tests, is inadvisable in the investigation of natural or experimental meningococcal disease, on account of the occurrence of IgA antibodies blocking the lytic effect of IgG and IgM [31]. Bactericidal antibody assay appears to be of little value in some important bacteriological diseases, e.g. brucellosis, the mycobacterioses and *Bordetella pertussis* infection. Two techniques are described below, the first a direct method of vibriocidal antibody assay, and the second an indirect radioisotopic method which depends on the release of ^{51}Cr from insulted cells.

Microestimation of vibriocidal antibody

Sera are first freed of bacteriological contamination. Suspensions of *Inaba* and *Ogawa* serotypes of *Vibrio cholerae* are prepared from cultures on heart-infusion agar and adjusted in sterile saline at 4° C to 10 opacity units of the International Opacity Standard. Working suspensions at 1 unit are made in 1:5 chilled complement prepared in cold 0·85 per cent sterile saline. Doubling dilutions of sera under test are dispensed in 0·025 ml volumes by any convenient procedure up to 1:1280, the same volumes of vibrio-complement being added from the cold. Tube controls include those for anti-complementary activity, plus appropriate positives and negatives in microtitre plates. After 1 hour's incubation at 37° C, 0·15 ml of heart-infusion broth is added to each well. All test plates are maintained at this temperature. Simultaneously broth additions (see Table 39.1) are made to the complement control tubes. Sensitized sheep erythrocytes are now added to the anti-complementary controls. Within 4 hours the complement controls usually exhibit an optical transmittance of 60–70 per cent, in which case the test wells are examined for turbidity. The end-point is normally well defined, vibriocidal levels being indicated by a transparent clarity of the well fluid. The whole system is refrigerated overnight, readings being confirmed next day. Where the anti-complementary control wells do not exhibit early (i.e. within 30 min) haemolysis, appropriate test serum titrations are called for in order that the degree of vibriocidal antibody masked by the serum anti-complementary activity might be assessed. Anti-complementary activity present in stored sera from

TABLE 39.1. Vibriocidal antibody determination by microtechnique

Material	Test		Controls					
			positive		complement		anticomplementary	
serum 1:5	—	—	—	—	—	—	0·025	0·025
blood 1:10–1:1280	0·025	0·025	—	—	—	—	—	—
positive control serum, 1:10–1:1280	—	—	0·025	0·025	—	—	—	—
complement 1:5 *V. cholerae** (*Ogawa*) 1·0 opacity unit	0·025	—	0·025	—	0·5	—	0·025	—
complement 1:5 *V. cholerae** (*Inaba*) 1·0 opacity unit	—	0·025	—	0·025	—	0·5	—	0·025
saline	—	—	—	—	0·5	0·5	—	—
heart infusion broth	0·15	0·15	0·15	0·15	3·0	3·0	—	—
sensitized sheep erythrocytes	—	—	—	—	—	—	0·025	0·025

* Cholera Research Laboratories strains, viz: *Ogawa* CRL 465, *Inaba* CRL 22463

human cholera infection, when tested against London School of Hygiene and Tropical Medicine strains, was detected in less than 1 per cent of samples. Direct vibriocidal antibody estimations are highly reproducible and are more sensitive than agglutination procedures. Sensitivity may be enhanced by the proportionate reduction of bacterial mass used in the test, followed by estimates of viable organisms. Recent vaccination, the type of vaccine used, the strains of test *Vibrio* employed, the infrequent (1 per cent) occurrence of false positive results, and contamination of sera under test are among factors to be considered in interpretation.

Radiovibriolysis

The release of radiochromium from labelled cells is an established index in estimation of immune damage *in vitro* [10] (see Chapter 36). The technique of radiobacteriolysis of gram-negative bacteria employs principally carbon and phosphorus radioisotopes [42] or, in the technique described here, ^{51}Cr [6]. In the presence of specific antibody plus complement, radiochromium is rapidly released from ^{51}Cr-labelled *Vibrio cholerae* cells.

Preparation of a standard immune serum
A group of rabbits is first bled to prepare a stock of normal serum (NS). The NS is inactivated at 56° C for 30 min, absorbed using sheep erythrocytes, filtered and stored at −20° C. The same rabbits are then given heat-killed (56° C, 2 hours) vibrios twice weekly intravenously in increasing doses on six occasions. Sera obtained after 1 month are pooled and prepared as for the NS pool.

Reference serum standards for vibriocidal antibody
Pools of sera from convalescent patients are in current use as reference standards for titrations of vibriocidal antibody (e.g. of the National Institute of Allergy and Infectious Diseases, USA). The vibriocidal titre of each pool is determined by means of the direct microtechnique and allocated a titre in units. This enables comparisons to be made between techniques and laboratories, but it should be observed that differences in titres obtained using radiovibriolysis and direct vibriocidal assay are considerable.

Vibrio ^{51}Cr labelling
Brain–heart infusion broth (BH) is inoculated with *V. cholerae* cells taken from an overnight culture on solid medium. After incubation with vigorous shaking for 1 hour at 37° C, the cells are washed in 0·01 per cent peptone:0·85 per cent saline (PS). The vibrios are resuspended in PS to a density of 10^9–10^{10}

organisms in 1 ml. To this volume $Na_2{}^{51}Cr\ O_4$ is added to a final concentration of 100–200 μCi per ml before incubation, with shaking as before. After rewashing in PS the cells are resuspended to 10^6–10^8 organisms per ml when required for bactericidal antibody assays. Labelling takes less than 3 hours by this procedure.

Assay procedure

Complement (C′) is diluted in cold PS, test-serum dilutions being made in C′PS at 4° C in 0·5 ml volumes. Aliquots of ^{51}Cr-labelled vibrios are then added and the tubes shaken at 37° C for 30 min. Centrifugation at 6000 × *g* for 10 min at 4° C follows, and 0·5 ml volumes are removed for duplicate radioassays. The necessary controls include (a) labelled vibrios in PS alone; (b) labelled vibrios and C′; (c) labelled vibrios and homologous antiserum prepared as above, and (d) labelled vibrios plus NS and C′. After correction for background, ^{51}Cr release is calculated from the radioactivity counts obtained:

$$\text{specific } {}^{51}\text{Cr release per cent equals}$$

$$\frac{\text{(counts per minute released in the presence of a particular dilution of immune serum and complement)} \ \textit{minus}\ \text{(counts per minute released in the presence of the same dilution of NS plus C′).}}{\text{(total counts per minute present in cells)} \ \textit{minus}\ \text{(counts per minute released in the presence of the same dilution of NS plus C′).}} \times 100$$

The titre, ^{51}Cr R_{50}, defined by Blachman is the reciprocal of that serum dilution yielding 50 per cent of the maximum specific radiochromium release.

The sensitivity of the assay diminishes as the vibrio cell mass is increased, the radiovibriolytic titre being inversely proportional over the range 10^6–10^9 vibrios per ml. The duration of incubation does not appear critical within the stated limits. Longer times, however, are to be avoided since ^{51}Cr-labelled cells in NS with a 1:20 dilution of C′ release at the 5-hour point high-release percentages comparable to equivalent dilutions of Standard Immune Serum. The specificity of radiovibriolysis can be demonstrated by comparing data obtained when organisms not normally cross-reactive with *V. cholerae* are similarly examined, e.g. *Aeromonas hydrophila* and *Escherichia coli* strains. The temperatures at which the procedures are carried out affects results, a reduction to 4° C from 37° C for the 30-minute assay lowering the percentage ^{51}Cr release by approximately one-half. Doubling the centrifugation times appears not to affect results significantly. The selected growth-phase point of the *V. cholerae* is a critical determinant of ^{51}Cr uptake, since *Vibrio* in the logarithmic phase assimilate additional amounts of ^{51}Cr when suspended in a fluid medium not permitting further cell division. The ^{51}Cr uptake during 1 hour using $Na_2{}^{51}Cr\ O_4$ is linear across a 10–100 μCi per ml range of chromate ion concentration.

Counter immunoelectrophoresis

The continuous (CIE) or discontinuous (DCIE) techniques used in bacteriology are subject to more procedural adaptations of technique than in other disciplines, on account of the range of organisms and the variety of antigens available. Running times vary between 30 (*Haemophilus influenzae b* free antigens; DCIE) and 120 min (*Clostridium tetani* antibody). In most studies a pH of 8·2–8·6 suffices. Exceptions are Kimble-Anderson reversed assays of *Staphylococcus* entertoxins where pH 6·0 in Tris-maleate buffer is employed. *Streptococcus pneumoniae*, *Corynebacterium diphtheriae*, *Neisseria meningitidis* and *Vibrio cholerae* have all been investigated by CIE. The technique has been used to detect cell fragments, toxins, diffused antigens in CSF and blood, and in antibody assay. The efficacy of DCIE is indicated by the fact that this procedure will detect as little as 0·2 ng antigen in CSF, even where several antigens are found together.

The adaptation of DCIE to the detection of *S. pneumoniae* capsular antigens in sputum [26], enables a more accurate interpretation of other bacteriological findings [60] in pneumococcal and other chest disease. It also points to the need for a re-examination of much epidemiological data, and suggests the advisability of incorporating DCIE into routine use for rapid and more accurate clinical diagnoses.

DCIE in detection of S. pneumoniae *capsular antigens*

Viable *S. pneumoniae* typed by standard procedures are grown in fluid culture. Streptococcal antigens are extracted by deproteinization and precipitation with acetone. They are redissolved in neutral distilled water before being stored in the frozen state. Test sputa are similarly processed and may be treated with a commercial mucolytic agent prior to being stored in the cold for batch examination. Agarose 1 per cent in veronal-acetate buffer, pH 6·6 and ionic strength 0·05, is used to coat glass slides to a depth of 1 mm. Two-microlitre quantities are employed, antibody wells being to the anodic side. The runs