Rapid Assays for the Detection and Determination of Sparse Populations of Bacteria and Bacteriophage T7 with Radioactively Labelled Homologous Antibodies

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SUMMARY

Small numbers of several bacterial species were rapidly and specifically detected with the ^{125}I -labelled antibody method described previously. Multibacterial species detection was achieved in one operation with ^{125}I -labelled mixtures of type-specific anti-bacterial globulins but the level and variability of the blank value increased, and sensitivity decreased, with the number of globulin components in the mixture. An indirect radio-assay that involved successive treatments of bacteria with unlabelled rabbit anti-bacterial serum and ^{125}I -labelled immunopurified goat anti-rabbit globulin was less sensitive and reproducible than the direct radio-assay. A modified assay, developed to detect and determine bacteria filtered on to a membrane filter, allowed the detection of small numbers of bacteria in large volumes of aqueous sample.

The feasibility of rapidly and specifically detecting small numbers of virus particles with ^{125}I -labelled anti-viral globulin was investigated with bacteriophage T7 as a model particle. Methods for the rapid separation of phage- ^{125}I -labelled globulin complex were developed and a minimum of about 5×10^5 total phage particles was detected.

INTRODUCTION

The rapid specific detection and determination of small numbers of *Escherichia coli* bacteria and *Bacillus subtilis* spores with ^{125}I -labelled homologous antibodies was reported by Strange, Powell & Pearce (1971). Application of the assay to the determination of several other bacterial species showed that, in general, a minimum of 10^3 to 5×10^3 bacteria could be detected. The potential sensitivity of the assay is much higher but experimental variations in test and blank responses swamp the signal given by smaller numbers of bacteria.

Sensitive fluorescent antibody-membrane filtration techniques have been reported for microbial detection (Danielsson, 1965; Danielsson & Laurell, 1965; Closs, 1968; Guthrie & Reeder, 1969; Jost & Fey, 1971) and 'staining' bacteria on membrane filters with radio-labelled antibody was investigated as a means of improving sensitivity. Initially, results were unsatisfactory because after treatment of a membrane filter with labelled antibody followed by extensive washing, the level of radioactivity retained by the filter was high and variable, and prevented detection of a small number of bacteria. A method of decreasing this interference was found and a small number of bacteria could be detected in a large volume of aqueous sample.

A specific immunological assay allows only one species of organism to be detected and estimated at a time and if samples contain one or more of several bacterial species, detection and specific identification depends on separate assays with each of the labelled homologous

antibodies. The possibility that detection (without immediate identification) of bacteria in such samples could be achieved in one operation was investigated with a direct method using a mixture of labelled antibodies and an indirect method based on the 'anti-globulin test' (Coombs, Mourant & Race, 1945). Bacteria could be detected with both methods but the indirect method was relatively insensitive.

The feasibility of detecting viruses with radioactively labelled homologous antibodies was investigated using bacteriophage T7 as a model particle in the appropriate size range. Data obtained for antibody taken up by phage T7 particles, compared with that taken up by bacteria, showed that sensitivity would be several orders of magnitude lower in terms of the minimum number of particles detectable but similar in terms of the minimum mass detectable. The rapid quantitative separation of phage-labelled antibody complex presented a problem but tentative methods of achieving this were devised and relatively small numbers of phage particles were detected.

METHODS

Micro-organisms. Washed suspensions of *Escherichia coli* serotypes O8K8, O8K9 (MRE 162) and O9K9, and viable *Bacillus subtilis* var *niger* spores purified by the method of Sacks & Alderton (1961) were prepared as described by Strange *et al.* (1971). *Serratia marcescens* (8 UK) grown in a peptone-mannitol medium was separated and washed ($\times 3$) by centrifugation with 0.85 % NaCl + 0.1 % formaldehyde and resuspended in 0.85 % NaCl. Suspensions of the 'Live Vaccine Strain' (LVS) of *Pasteurella tularensis* (Tigertt, 1962; Sharer, Klein & Lincoln, 1968) were provided by our colleague Mr C. G. T. Evans. Formalin-killed suspensions of *P. pestis* (M3) and *Brucella* species were provided by our colleague Mr F. C. Belton.

A suspension of bacteriophage T7 (10^{11} to 10^{12} plaque-forming units (p.f.u.)/ml) was provided as a crude lysate by Microbial Products Section, Microbiological Research Establishment (MRE). Phage was purified by a method similar to that described by Isenberg (1963) for the purification of phage μ_2 . Plaque-forming unit (p.f.u.) counts on suitably diluted samples on lawns of *Escherichia coli* (MRE 160) and counts under the electron microscope, indicated that 20 to 60 % of phage in the lysates was recovered in purified preparations.

Assessment of bacterial and phage suspensions. Suspensions of viable bacteria were assessed with colony counts on nutrient agar plates, total counts in a Thoma chamber (Hawksley, London) and dry weight determinations (bacteria washed with distilled water and dried at 103 °C for 18 h). Suspensions of killed bacteria were assessed with dry weight determinations and, provided that the bacteria had not aggregated, with total counts.

Suspensions of phage T7 were assessed by plaque counts (Adams, 1959) on lawns of *Escherichia coli* MRE 160, total particle counts and dry weight determinations. The total number of phage particles in samples was determined by our colleague, Mr W. J. Harris; a known concentration of graded polystyrene latex particles (diam. 0.109 μ m; Dow Chemical Co., Midland, Michigan, USA) was added to a suitably diluted sample and differential counts made after negative staining with phosphotungstic acid under a Siemens Elmiskop IA electron microscope with a magnification of $\times 102600$. Phage dry weight was determined by sedimenting phage from a measured volume of suspension in an ultracentrifuge (Spinco model L; 34000 rev./min, 2.5 h), washing the pellet by resuspension in saline phosphate buffer (pH 7.7) diluted 1 in 10 with water and recentrifugation, drying the pellet at 103 °C and weighing.

Solutions. Saline phosphate buffer contained 0.11 M-NaCl and 0.02 M-phosphate (Na; pH 7.7) in glass distilled water. 'Saline phosphate buffer + Brij 35' contained 0.1 or 1 % (w/v) polyoxyethylene lauryl ether (Brij 35; British Drug Houses, Ltd, Godalming Surrey).

Solutions were filtered through Millipore membrane filters ($0.22\ \mu\text{m}$) immediately before use.

Filters. Membrane filters tested for radio-assay purposes were Millipore mixed cellulose ester (MF) and cellulose acetate (Celotat^(R)) with pore sizes of 0.22 , 0.3 and $0.45\ \mu\text{m}$ (Millipore Ltd, Park Royal, London, N.W.10); Sartorius cellulose nitrate, cellulose acetate and polyvinyl chloride (0.2 and $0.45\ \mu\text{m}$; V. A. Howe & Co. Ltd, London, W.11); Flotronics silver ($0.45\ \mu\text{m}$; Sela Flotronics, Spring House, Pennsylvania 19477, USA); Nuclepore polycarbonate ($0.5\ \mu\text{m}$; General Electric Co, Pleasanton, California 94566, USA). Discs of Whatman glass fibre paper (GF/C; H. Reeve Angel & Co. Ltd, London, E.C.4) were also tested.

Filter holders. Stainless steel filter jigs as described by Strange *et al.* (1971) fitted with membrane filters (2 cm diam.) were used in the radio-assay. Millipore Swinny stainless steel filter holders (13 mm diam.) fitted with membrane filters ($0.22\ \mu\text{m}$) and a hypodermic syringe (5 ml) were used for filtering labelled antibody reagents into test samples. A Millipore stainless 47 mm pressure filter holder, fitted with a series of filters of decreasing pore size down to $0.22\ \mu\text{m}$, was used for filtering antiserum for the production of purified antibody.

Purification of antibodies. A general method for the immunopurification of anti-bacterial globulins was described previously (Strange *et al.* 1971) and a similar method was used for purifying anti-phage T7 globulin. Antiserum (20 ml; precipitated phage T7 at a dilution of 1/512 to 1/1024) from rabbits immunized with purified phage T7 was adsorbed three times over a period of 24 h with *Escherichia coli* (MRE 160) (equiv. 120 mg dry wt) to remove host cell antibodies. Absorbed serum was freed from bacteria by centrifugation and filtration through a Millipore membrane filter ($0.22\ \mu\text{m}$). Purified phage suspension (2 ml, 7×10^{12} p.f.u.) was added dropwise with mixing to the antiserum and the suspension stirred for 3 h at 20°C . After standing for 16 h at 2°C the immune complex was separated and washed ($\times 3$) with 0.85% NaCl by centrifugation (5000 g, 15 min) and then extracted twice with $0.1\ \text{M-HCl} + 1\%$ NaCl (pH 1.2; 3 ml) for 15 min at 0°C with brief agitation on a Whirlimixer (Fisons Scientific Apparatus, Loughborough, Leicester). Extracts were separated by centrifugation (5000 g, 30 min) pooled and adjusted (M-NaOH) to about pH 6.0. The small precipitate was removed by centrifugation and the clear supernatant treated with an equal vol. of saturated $(\text{NH}_4)_2\text{SO}_4$ solution. After 1 h at 2°C precipitate was separated, dissolved in saline phosphate buffer (1 ml) and reprecipitated with $(\text{NH}_4)_2\text{SO}_4$. After a second reprecipitation, the final product was dissolved in saline phosphate buffer (1 ml) and dialysed against the same buffer until free of detectable ammonium salt. The dialysed product was centrifuged to remove insoluble material and stored at -15°C . The yield of soluble protein was 3 to 4.8 mg with a specific precipitating activity of 50 units (reciprocal of maximum dilution precipitating phage)/mg protein/ml.

Purified goat anti-rabbit globulin was prepared by adsorbing goat anti-rabbit serum with normal rabbit globulins, polymerized with glutaraldehyde (Avrameas & Ternynck, 1969), and eluting the immune complex with $0.1\ \text{M-HCl} + 1\%$ NaCl (pH 1.2). Protein in the neutralized eluate was precipitated with $(\text{NH}_4)_2\text{SO}_4$ (50% saturation), redissolved in saline phosphate buffer and dialysed against the same buffer until free of detectable ammonium salt. The yield of soluble protein was about 3 mg. Quantitative precipitin tests (Kabat & Mayer, 1961) showed that the immunological activity of the purified antibody was only eight- to tenfold that of the parent antiserum. This was a relatively low purification factor, but no higher specific immunological activity was obtained with immunoadsorbents of rabbit globulins polymerized with polystyrene (Kent & Slade, 1960) or 'Immunothiol' (Stephen, Gallop & Smith, 1966).

Radioactive labelling of antibodies. Immunopurified globulins were usually iodinated with ^{125}I using the method of Hunter & Greenwood (1962) as previously described (Strange *et al.* 1971). The methods of Glover, Salter & Shepherd (1967) and McFarlane (1956; 1958) were also tested to see whether the labelled products were more suitable for radio-assay purposes. Also a comparison of ^{131}I and ^{125}I as antibody labels was made. After labelling (2 to 80 $\mu\text{Ci}/\mu\text{g}$ globulin), antibody protein (10 to 20 $\mu\text{g}/\text{ml}$) was mixed with membrane-filtered (Millipore, 0.22 μm) homologous normal serum (1/6th final volume) and stored at 4 °C.

Radio-assay procedures. The standard method involved filtration and washing (saline phosphate buffer + 1 % Brij 35) reaction mixtures of bacteria and ^{125}I -labelled antibodies on Millipore membrane filters (0.45 μm ; 2 cm diam.) as previously described (Strange *et al.* 1971).

Bacteria filtered on to membrane filters were detected as follows. The filter disc (2 cm diam.) was placed on a circular trough (1.5 cm diam.) cut into gelled agar (1.5 %, w/v, in saline phosphate buffer + 1 % Brij 35) and filled with buffer + surfactant solution, and a disc of washed filter paper (7 mm diam.) placed on the membrane centre. ^{125}I -Labelled antibody reagent (10 to 20 μl) was added to the paper disc and allowed to react for 1 to 4 min at 20 °C. Labelled antibody was thus localized and saturation of the membrane filter with surfactant solution decreased combination of labelled antibody with membrane material. After reaction, the paper disc was discarded and the membrane filter placed on several layers of washed filter paper to absorb excess antibody; 2 to 3 small vol. (10 μl) of surfactant solution were then applied and allowed to soak through. Finally, the membrane filter was transferred to a filter jig and thoroughly washed with saline phosphate buffer + 1 % Brij 35 by suction. Control membrane filters were treated in parallel with each series of tests and the radioactivities of the punched out filtration areas (7 mm) of the test and control filters were measured.

In the indirect radio-assay: (i) samples (0.1 to 1 ml) of bacterial suspension and buffer alone were incubated with homologous rabbit anti-bacterial serum (0.05 to 0.1 ml) at 25 °C; (ii) the immune complex was separated and washed ($\times 2$) with saline phosphate buffer (pH 7.7) by centrifugation; (iii) the complex was resuspended in buffer (0.1 ml) and incubated with ^{125}I -labelled goat anti-rabbit globulin reagent (0.05 to 0.1 ml; 0.5 to 1 μg antibody protein; 20 to 40 $\mu\text{Ci}/\mu\text{g}$) containing one sixth vol. of normal goat serum; (iv) the reaction mixture was filtered and washed on a Millipore membrane filter (0.45 μm pore size; 2 cm diam.); (v) the radioactivities of the punched out filtration areas of the test and control membrane filters were measured. In addition, controls of bacteria not treated with anti-bacterial serum but treated with ^{125}I -labelled goat anti-rabbit globulin were included.

Three assay procedures for determining phage T7 were developed. The first involved adsorption of phage on to bacteria; exponential phase *Escherichia coli* MRE160 cells were grown for 2 to 3 h at 37 °C in shaken flasks of nutrient broth heavily seeded with a fully grown overnight culture in the same medium. The bacteria were separated and washed ($\times 2$) with 0.85 % NaCl by centrifugation and resuspended (equiv. 0.25 to 0.5 mg bacterial dry wt/ml) in 0.85 % NaCl. Samples (0.1 ml) of phage suspension (5×10^5 to 10^7 p.f.u./ml) in membrane-filtered (Millipore, 0.22 μm) nutrient broth and broth alone were incubated with washed bacterial suspension (0.025 ml; equiv. 6 to 12 μg bacterial dry wt) for up to 15 min at 37 °C. ^{125}I -Labelled immunopurified anti-phage globulin reagent (50 μl ; 0.5 to 1 μg antibody protein; 20 to 80 $\mu\text{Ci}/\mu\text{g}$) containing one sixth vol. normal rabbit serum was added and the suspension incubated at 25 °C for up to 15 min. Test and control reaction mixtures were filtered and washed on Millipore membrane filters (0.45 μm) and the radioactivities of the punched out filter centres (7 mm diam.) measured.

The second procedure involved 'immunofiltration'. The centres of Millipore membrane

filters ($0.45\ \mu\text{m}$; 2 cm diam.) were treated with 10 to 20 μl immunopurified anti-phage globulin solution (1 to 4 mg antibody protein/ml saline phosphate buffer, pH 7.7) and heated at $60\ ^\circ\text{C}$ for 30 min to fix the protein. The volume of antibody solution applied was sufficient to impregnate the filtration area of the filter. Reaction mixtures (0.1 to 0.2 ml) of phage suspension or buffer alone, and ^{125}I -labelled anti-phage globulin reagent were filtered and washed on the impregnated filters and the radioactivities of the punched out filter centres measured. Results were improved by placing an untreated Millipore membrane filter ($0.45\ \mu\text{m}$; 2 cm diam.) over the impregnated membrane; after filtering and washing a reaction mixture on both membranes, the top membrane was discarded.

The third procedure involved breakage of phage by freezing and thawing in distilled water before reaction with ^{125}I -labelled antibody. Samples (1 ml) of phage suspension in water were quickly frozen and thawed at least six times by immersion in solid CO_2 + acetone and water at $37\ ^\circ\text{C}$, respectively. ^{125}I -Labelled anti-phage globulin reagent (0.05 to 0.1 ml) and double strength saline phosphate buffer (pH 7.7; 0.1 ml) were then added to the phage suspension (0.1 ml) and to water controls. After incubation at $25\ ^\circ\text{C}$ for the selected reaction time, the reaction mixtures were filtered and washed on Millipore membrane filters ($0.22\ \mu\text{m}$; 2 cm diam.) and radioactivities measured in the usual way. Higher signals were obtained if, after incubation with ^{125}I -labelled anti-phage globulin, an excess of membrane-filtered anti-phage serum (0.1 ml) was added and incubation continued for a few min before filtration.

Measurement of radioactivity. Samples labelled with ^{125}I were usually counted with a well-type sodium iodide crystal scintillation counter and scaler (Spectrometer NE 8622, Nuclear Enterprises, Edinburgh). To compare the effectiveness of ^{125}I and ^{131}I as labels for antibody proteins, samples were counted in a suitably programmed automatic liquid scintillation analyser (Model PW 4510; N. W. Philips, Gloelampenfabrieken, Eindhoven, The Netherlands) and counts converted to d.p.s. according to the respective counting efficiencies. The counting vials and scintillation fluid described by Ashcroft (1970) were used to count ^{125}I attached to membrane filter discs (7 mm diam.).

Samples were determined at least in duplicate and the mean of a number of blank samples (no micro-organisms) was subtracted from the mean test value to give the 'signal'.

Analytical methods. Protein was determined by ultraviolet measurements at 260 and 280 nm (Warburg & Christian, 1942) and the method of Lowry, Rosebrough, Farr & Randall (1951) with dried bovine γ -globulin (Cohn fraction 11; Sigma Chemical Co., Kingston upon Thames, Surrey) as standard.

RESULTS AND DISCUSSION

Sensitivity of direct ^{125}I -labelled antibody methods for detecting bacteria

Factors affecting the sensitivity of the direct radio-antibody assay for detecting bacteria are the rate of uptake of antibody, the specific radioactivity of antibody protein, the reaction time and temperature, and the composition of the suspending medium. The rate of uptake of antibody depends on antibody concentration and the maximum amount of antibody taken up depends on the amount and accessibility of homologous antigen on the bacterial surface. When *Escherichia coli* MRE162 cells were exposed to an excess of homologous antiserum, the maximum amount of antibody taken up was equivalent to at least 20 % of the bacterial dry weight (based on the results of quantitative agglutination tests; Kabat & Mayer, 1961). For assay purposes, however, time of reaction of bacteria with antibody was kept to a minimum and labelled antibody concentration limited to control the level and variability of the assay blank value. Under these conditions, the amount of antibody taken up did not

Table 1. Mean uptake of ^{125}I -labelled anti-K9 globulin by individual cells of *Escherichia coli* serotype O8K9 (MRE162)

Duplicate samples (1.1 ml) containing 6×10^3 to 10^4 bacteria and saline-phosphate buffer alone were incubated with ^{125}I -labelled anti-K9 globulin (4.2 μg ; 8.6 $\mu\text{Ci}/\mu\text{g}$) and normal rabbit serum (3 %, v/v) for 4.5 min at 22 °C. Reaction mixtures were filtered and washed (saline-phosphate buffer + 1 % Brij 35) on Millipore filters (0.45 μm ; 2 cm diam.) and radioactivities of the filter centres (7 mm) measured. The mean assay blank value was subtracted from the mean test counts. ^{125}I -Labelled anti-K9 protein (1 ng) gave 76.8 c.p.s.

No. bacteria	6000	8000	10000
Signal (c.p.s.)	13.2	16.4	19.8
Antibody uptake (ng)	0.172	0.214	0.258
Antibody uptake (molecules)	6.5×10^8	8.1×10^8	9.8×10^8
Molecules antibody per bacterium	1.08×10^5	1.0×10^5	0.98×10^5
Bacterial dry wt (ng)	1.5	2.0	2.5
Antibody uptake (% dry wt)	11.5	10.7	10.3

approach saturation level. For example, in the presence of 4.2 μg ^{125}I -labelled anti-K9 globulin/1.1 ml reaction mixture, 6×10^3 to 10^4 *E. coli* cells combined with antibody equivalent to only about 10 % of bacterial dry weight and the mean number of antibody molecules (assumed mol. wt 160000) attaching to a single bacterium was about 10^5 (Table 1). If antibody protein is labelled with 1 atom ^{125}I /mol and the efficiency of counting ^{125}I is 40 %, 10^5 antibody molecules attached to a bacterium will give a signal of about 0.32 c.p.m. The mean radioactive counting instrument background was 16 c.p.m. (S.D., ± 3 c.p.m.) and if a signal equivalent to twice the instrument background is considered significant, about 100 bacteria could be detected. Since the specific radioactivity of antibody protein can be increased up to about 3 atoms ^{125}I /mol with a proportional increase in signal/bacterium, about 30 bacteria should be detectable. In practice, however, less than about 500 *E. coli* cannot be detected with the assay because labelled immune complex is not completely separated from all non-specifically combined radioactivity.

Assay blank value. Methods tested for separating labelled immune complex from a reaction mixture included centrifugation, electrophoresis, chromatography and membrane filtration. The latter method was adopted for speed and convenience but, after filtration of a solution of ^{125}I -labelled globulin and washing, the membrane filter was radioactive to an extent depending on the membrane material, speed of filtration, washing conditions and the properties of the antibody globulin. Membrane filters of various materials (see Methods) were tested for retention of radioactivity after filtration of samples (1 ml) of labelled antibody reagent and a standard washing procedure. Values were extremely high with silver membranes, fairly high with cellulose acetate membranes, moderate with cellulose mixed ester, cellulose nitrate and polyvinyl membranes and lowest with Nuclepore polycarbonate membranes. Millipore mixed ester membrane filters (HA; black and white, 0.22 and 0.45 μm) were used routinely; the more expensive Millipore ultrathin membranes (25 μm thick) of the same material gave somewhat higher radioactivity retention values under the conditions used.

Washing solutions containing a variety of sodium salts (Cl^- , PO_4^{3-} , CO_3^{2-} , HCO_3^- , I^- , OH^- , acetate, barbiturate), iodine, tris, serum proteins, ^{127}I -labelled globulins and/or various surfactants at pH values of 6 to 12 tested for their ability to remove residual radioactivity from a membrane filter. Saline phosphate buffer (pH 7.7) containing 1 % (w/v) Brij 35 was among the most efficient washing solutions and tests showed that as used in the assay, it did not cause significant dissociation of bacterial immune complexes. 'Decon' (Medical

Table 2. Proportion of ^{125}I -labelled immunopurified anti-*Bacillus subtilis* spore globulin taken up by an excess of spores

Iodination procedures were those of (1) Hunter & Greenwood (1962), (2) McFarlane (1956) and (3) Glover *et al.* (1967). Antibody uptake was determined by incubating purified spores and ^{125}I -labelled antibody for 1 to 2 h at 37 °C, holding the mixture for 16 h at 2 °C (with mixing at intervals), separating the immune complex by centrifugation and comparing the radioactivity of a suitably diluted sample of supernatant fluid with that of similarly diluted control antibody solution. The absorption process was usually repeated with fresh spores.

Iodination procedure	Specific radio-activity of antibody ($\mu\text{Ci}/\mu\text{g}$)	Wt antibody (μg)	No. spores	No. exposures to spores	Total vol. (ml)	Uptake of ^{125}I (%)
(1)	3	15	4×10^8	$\begin{cases} 1 \\ 2 \\ 3 \end{cases}$	0.5	$\begin{matrix} 44 \\ 51 \\ 57 \end{matrix}$
(1)	8	5	3×10^8	$\begin{cases} 1 \\ 2 \end{cases}$	0.5	$\begin{matrix} 29 \\ 35 \end{matrix}$
(1)	8.5	5	3×10^8	1	0.5	39
(2)	2.0	5	3×10^8	1	0.5	31
(2)	2.0	7	2×10^8	$\begin{cases} 1 \\ 2 \end{cases}$	0.7	$\begin{matrix} 31 \\ 38 \end{matrix}$
(3)	2.0	7	2×10^8	$\begin{cases} 1 \\ 2 \end{cases}$	0.7	$\begin{matrix} 38 \\ 44 \end{matrix}$

Pharmaceutical Developments Ltd, Portslade, Brighton, Sussex) completely cleared radioactivity from membrane filters but bacterial immune complex was also dissolved away.

In addition to attachment of soluble labelled protein to membrane filters, labelled particles present in antibody reagents may be retained and contribute to the assay blank value. Filtration of antibody reagents immediately before use decreased but did not necessarily eliminate this interference because some aggregation occurred during the reaction period. Solutions of macroglobulin (IgM; mol. wt about 10^6 daltons) tended to autoprecipitate and were generally unsuitable. For example, when samples of ^{125}I -labelled horse anti-*Escherichia coli* MRE 162 IgM were filtered and washed on membrane filters, residual radioactivity was 50- to 100-fold that remaining when samples containing the same amounts of ^{125}I -labelled sheep IgG (mol. wt about 1.6×10^5 daltons) of the same specific radioactivity were similarly treated.

Purification of antibodies. A general method for the preparation of immunopurified globulins against bacteria described previously (Strange *et al.* 1971) was used to prepare antibodies against a number of bacterial species. Based on the agglutinating activity of the parent antiserum, yield of purified globulins was usually 10 to 25 %. Low yields were due mainly to losses of spontaneous precipitation of protein during processing presumably caused by denaturation. Soluble protein eluted from immune complex was separated into 2 or 3 fractions with $(\text{NH}_4)_2\text{SO}_4$ and fractions precipitating between 25 and 50 % saturation of salt were usually suitable for use in the radio-assay.

Vegetative bacteria killed with formaldehyde (0.1 to 0.3 %) for 24 h followed by washing with 0.85 % NaCl and the dissociating agent subsequently used to extract the immune complex, followed by further washings with 0.85 % NaCl, were usually satisfactory immuno-adsorbents. Or, in the case of bacteria with heat-resistant surface antigens, boiling suspensions in 0.85 % NaCl (about 10 mg bacterial dry wt/ml) under reflux for 1 to 2 h, washing with 0.85 % NaCl, extraction with dissociating agent and further washing with 0.85 % NaCl was

Fig. 1. Assay of *Escherichia coli* serotype O8K9 (MRE 162), *Serratia marcescens* (UK8) and *Pasteurella tularensis* (LVS) with ^{125}I -labelled homologous antibodies. Bacteria (10^3 to 10^6) in saline phosphate buffer (0.1 ml) were incubated at 25 °C with ^{125}I -labelled homologous antibody (1 μg anti-*E. coli* O8 globulin, 16 $\mu\text{Ci}/\mu\text{g}$ for 6 min; 0.5 μg anti-*S. marcescens* globulin, 23 $\mu\text{Ci}/\mu\text{g}$ for 6 min; 0.67 μg anti-*P. tularensis* globulin, 20 $\mu\text{Ci}/\mu\text{g}$ for 12 min). Reaction mixtures and controls were treated as in Table 1. *Escherichia coli* O8K9, Δ ; *S. marcescens* (UK8), $\circ$; *P. tularensis* (LVS) $\bullet$.

a suitable pretreatment. A good general agent for dissociating bacterial immune complexes was 0.1 N-HCl + 1 % (w/v) NaCl (pH 1.2).

The purity of antibody eluted from bacterial immune complexes depended on the concentration of bacteria used to adsorb antibody from homologous antiserum. Despite pretreatment of the bacteria, the dissociating agent eluted a small amount of cellular material from the immune complex that increased with bacterial concentration. The optimum concentration of bacteria was determined in a pilot experiment in which small volumes (1 ml) of antiserum were adsorbed with increasing concentrations of bacteria. The resulting immune complexes were separated and washed by centrifugation and treated with dissociating agent. The extracts were neutralized and tested for agglutinating activity and protein content. With *Serratia marcescens*, the optimum bacterial concentration for adsorbing homologous antiserum with an agglutination titre of 1 in 1024 was about 10 mg equiv. bacterial dry wt/ml; lower bacterial concentrations incompletely adsorbed antibody and higher concentrations gave products with lower $E_{280\text{ nm}}/E_{260\text{ nm}}$ values indicating increasing contamination of the eluted protein. Immunopurified antibody suitable for use in the radio-assay had a $E_{280\text{ nm}}/E_{260\text{ nm}}$ ratio of 1.7 to 1.9 (free of nucleic acids) and dissolved easily in saline phosphate buffer (pH 7.7) to give a clear solution at a concentration of at least 10 mg/ml.

Immunopurified antibody eluted from bacterial immune complexes contained molecules

Table 3. Detection of five bacterial species with ^{125}I -labelled mixture of individual type-specific antibodies

Samples (0.1 ml) of bacterial suspensions and buffer alone were incubated with ^{125}I -labelled mixed antibodies (0.1 ml containing 0.8 μg each of anti-*Brucella abortus* (BA), anti-*Escherichia coli* (EC), anti-*Pasteurella tularensis* (UL), anti-*Pasteurella pestis* (PP) and anti-*Bacillus subtilis* spore (BS) globulins; 8 μCi (μg) and normal rabbit serum (16 μl) for 6 min at 25 °C. Reaction mixtures were treated as in Table 1.

Test	Sample	c.p.s.		Detection limit (ng dry wt)
		Blank	Signal*	
1	Buffer	2.2	—	—
2	5ng BA		24.2	1.0
3	5ng EC		134.0	0.17
4	5ng UL		25.0	1.0
5	5ng PP		13.3	1.7
6	5ng BS		57.0	0.38
7	Mixture of bacteria as in tests 2 to 6		213.0†	—

* Blank subtracted.

† Sum of individual responses for tests 2 to 6, 254 c.p.s.

Table 4. Indirect radio-assay of *Pasteurella tularensis* (Schu 4) with two antibodies

Formalin-killed *Pasteurella tularensis* (equiv. 50 μg bacterial dry wt) were incubated with immunopurified anti-*P. tularensis* globulin (132 μg protein) for 30 min at 25 °C. The complex was separated and washed ($\times 2$) with saline-phosphate buffer (pH 7.7) by centrifugation and resuspended in buffer (10 ml). Portions (0.1 ml, equiv. 0.5 μg bacterial dry wt) were incubated with ^{125}I -labelled goat anti-rabbit globulin reagent (50 μl ; 1.8 μg antibody; 8 $\mu\text{Ci}/\mu\text{g}$ protein) for 18 min at 25 °C. The reaction mixtures were treated as in Table 1. Controls of saline phosphate buffer and samples of *P. tularensis* not treated with anti-*P. tularensis* globulin were tested in parallel. Results are means of duplicate tests.

Sample	Anti- <i>P. tularensis</i> globulin	^{125}I -Goat anti- rabbit globulin	c.p.m.	
			Response	Signal (3-2)
1 Buffer alone	+	+	1486	—
2 <i>P. tularensis</i> (0.5 μg *)	—	+	3860	—
3 <i>P. tularensis</i> (0.5 μg)	+	+	10172	6312

* Bacteria had aggregated and total counts could not be made; 0.5 μg dry wt is equivalent to about 2×10^6 cells.

with a number of immunological specificities according to the different antigens accessible on the bacterial surface. Different immunologically specific antibodies were not separated except in the case of *Escherichia coli* serotypes O9K9, O8K8 and O8K9 for which purified anti-O and anti-K globulins were prepared by adsorbing anti-serum with bacteria having either the homologous O or K antigen (Strange *et al.* 1971).

Immunopurified anti-bacterial globulins labelled with ^{125}I were not completely taken up by an excess of homologous bacteria indicating that they were impure, partially inactivated during preparation and/or damaged by iodination. For example, the maximum uptake of ^{125}I -labelled anti-*Bacillus subtilis* spore globulin by an excess of *B. subtilis* spores was 58 % and uptake decreased with increasing specific radioactivity of the antibody (Table 2). Using the chloramine-T iodination method, low uptake of labelled antibody could have been due to the action of the oxidant on antibody protein during iodination but decreasing the con-

Table 5. *Detection of Bacillus subtilis spores on membrane filters*

Samples of a suspension of unpurified spores and saline-phosphate buffer alone were filtered on Millipore filters ($0.45\ \mu\text{m}$; 2 cm diam.). Filters with 10^4 spores and controls were treated as described in the text with (a) ^{125}I -labelled anti-*B. subtilis* spore globulin reagent ($17\ \mu\text{l}$; $36\ \mu\text{g}$ antibody protein/ml; $6\ \mu\text{Ci}/\mu\text{g}$) for 1 to 4 min and (b) 10 to $25\ \mu\text{l}$ labelled antibody reagent for 3 min. Filters with 2×10^8 to 10^4 spores and controls were treated with (c) $17\ \mu\text{l}$ labelled antibody reagent for 2 min. The filters were washed (saline-phosphate buffer + 1% Brij 35) and radio-activities of the centres (7 mm) measured. Results are given in c.p.s.

(a) Reaction time with 10^4 spores (min)							
1		2		3		4	
Blank	Signal	Blank	Signal	Blank	Signal	Blank	Signal
9.5	81	11	101	9.5	112	12	112

(b) Amount of ^{125}I -labelled antibody (μg) with 10^4 spores for 3 min							
0.36		0.61		0.9			
Blank	Signal	Blank	Signal	Blank	Signal		
6	66	9.5	112	11	147		

(c) No. spores							
c.p.s.	0	2000	4000	6000	10000		
Signal (c.p.s.)	10.3	23.7	43.6	52.0	81.0		
	—	13.4	33.3	41.7	70.7		

centration of oxidant did not improve subsequent uptake; nor did use of the iodine chloride method of McFarlane (1956, 1958) improve subsequent uptake of labelled antibody (Table 2). The main reason for incomplete uptake of ^{125}I -labelled antibody by homologous bacteria was probably the damage caused by iodination.

Reproducibility of results. Detection of very small numbers of bacteria depends on the reproducibility of the assay blank value and replicate values determined with a number of ^{125}I -labelled antibodies showed that variations from the mean may be equivalent to the radioactive signal given by an appreciable number of bacteria. For example, the results for 16 blank determinations with ^{125}I -labelled anti-*Escherichia coli* K9 globulin ranged between 0.44 and 4.6 c.p.s.; mean, 1.8 c.p.s.; and S.D., 1.2 c.p.s. Since 10^4 *E. coli* cells gave a mean signal of 17.5 c.p.s., the maximum variation of 2.8 c.p.s. from the mean was equivalent to about 1.6×10^3 bacteria. Divergent results may arise because extraneous particulate matter is picked up from the environment and/or membrane filter material varies slightly.

Comparison of ^{131}I - and ^{125}I -labelled antibody for assay purposes. A possible disadvantage of ^{125}I compared with ^{131}I is that more iodine atoms must be attached to antibody molecules to achieve a given specific radioactivity and the chance that active sites will be inactivated is greater. If, however, active sites are not seriously affected, labelling antibody to contain a mean of more than one atom iodine/molecule protein may be advantageous since all molecules are likely to contain at least one atom of radioisotope and unlabelled antibody will not preferentially attach to bacteria.

Samples of anti-*Bacillus subtilis* var *niger* globulin were labelled to about the same specific radioactivity with ^{125}I and ^{131}I , respectively, and used to assay purified *B. subtilis* spores. The results showed that 500 spores were detectable and gave similar counts with each labelled antibody. Since ^{131}I -labelled antibodies lost immunological activity more rapidly than ^{125}I -labelled antibodies, the latter are more suitable for the radio-assay.

Table 6. Radio-assay of phage T7 attached to *Escherichia coli* (MRE 160)

(a) Phage T7 suspension (0.1 ml; 10^7 p.f.u.) in membrane-filtered broth was incubated with *Escherichia coli* MRE 160 (25 μ l suspension; 6 μ g bacterial dry wt) for 6 to 15 min at 37 °C. 125 I-labelled anti-phage globulin reagent (0.1 ml; 1.2 μ g antibody protein; 40 μ Ci/ μ g) was added and the mixture incubated at 25 °C for 15 min.

(b) As for (a) but phage was incubated with bacteria for 15 min and attached phage with labelled antibody for 6 to 15 min.

(c) Phage suspension (0.1 ml; 5×10^5 to 10^7 p.f.u.) was incubated with bacteria for 15 min and attached phage with labelled antibody reagent (0.05 ml; 0.5 μ g antibody protein; 80 μ Ci/ μ g.) for 15 min.

Reaction mixtures were treated as in Table 1.

(a) Incubation time				
with bacteria (min)	6	9	15	
Assay blank (c.p.s.)	2.8	4.9	3.5	
Signal (10^7 phage p.f.u.) (c.p.s.)	34.0	45.4	107.8	
(b) Incubation time with				
labelled antibody (min)	6	9	15	
Assay blank (c.p.s.)	3.6	4.0	6.5	
Signal (10^7 phage p.f.u.) (c.p.s.)	52.4	86.4	147.7	
(c) No. phage p.f.u.				
	0	5×10^5	10^6	10^7
c.p.s.	11.5	56.5	93.5	217.5
Signal (c.p.s.)	—	45.0	82.0	206.0

Detection and determination of different bacterial species

Assay of *Escherichia coli* serotypes O8K9 and O9K9 with 125 I-labelled anti-K9 globulin was reported previously (Strange *et al.* 1971). *Escherichia coli* serotypes containing O8 globulin can also be assayed with labelled anti-O8 globulin but sensitivity (a minimum of 5×10^3 bacteria detected) was lower than with anti-K9 (Fig. 1).

The minimum number of *Serratia marcescens* (UK8) or *Pasteurella tularensis* detected with labelled antibody was 2.5 to 5×10^3 (Fig. 1). Formaldehyde-killed *Brucella abortus*, *B. melitensis* and *B. suis* were all detected with labelled anti-*B. abortus* (544) globulin (7.2 μ g/ml; 9 μ Ci/ μ g) but sensitivity was relatively low and varied with species; the minimum numbers detectable with a 6 min reaction time at 25 °C were about 2×10^4 , 4×10^4 and 3×10^4 , respectively. Smaller numbers of *Brucella* species might be detected with longer reaction time and/or antibody of higher specific radioactivity.

Multi-bacterial species detection

Direct assay. Labelled mixed antibody reagents were prepared either by mixing pre-labelled antibodies or labelling a mixture of antibodies. Individual antibodies were not necessarily equivalently labelled with the latter method, i.e. certain antibodies took up more iodine than others. Each component in the mixture had to be labelled to a specific radioactivity commensurate with the sensitivity required for detecting the homologous bacterial species and the radioactivity of the reagent increased with the number of antibody components. As the radioactivity of the reagent increased, the assay blank value increased and sensitivity decreased. To detect a minimum of about 10^4 bacteria in 1 ml samples with a 125 I-labelled 5 antibody component mixture and reaction time of 6 min, each sample had to be treated with at least 20 μ g mixed antibody proteins combined with at least 130 μ Ci 125 I. The amount of labelled reagent added can be decreased by concentrating samples before assay, for example, by centrifugation or filtration of rapidly stirred samples through membrane filters (Strange *et al.* 1971).

Table 7. *Detection of phage T7 with a radioactively-labelled antibody-immunofiltration method*

(a) Samples of phage suspension (0.1 ml; 10^7 p.f.u.) were incubated with ^{125}I -labelled anti-phage globulin reagent (0.05 ml; 0.5 μg antibody protein; 40 $\mu\text{Ci}/\mu\text{g}$) for 15 min at 25 °C. Reaction mixtures and controls were filtered and washed on Millipore filters (0.45 μm ; 2 cm diam) on top of similar filters impregnated with heat-fixed anti-phage globulin (5 to 38 μg).

(b) Samples of phage suspension (0.1 ml; 10^7 p.f.u.) were incubated with antibody reagent (0.05 ml; 0.25 μg antibody protein; 34 $\mu\text{Ci}/\mu\text{g}$) for 6 to 15 min at 25 °C; reaction mixtures were filtered and washed through filters impregnated with 10 μg heat-fixed anti-phage globulin.

(c) Samples of phage suspension (0.1 ml; 5×10^5 to 10^7 p.f.u.) were incubated with labelled antibody reagent as in (a); reaction mixtures were filtered and washed through membranes impregnated with 20 μg heat-fixed antibody. The radioactivities of the impregnated filter centres (7 mm diam.) were measured.

(a) Heat-fixed antibody in membrane (μg)	5	10	20	38
Blank (c.p.s.)	8	9	19	92
Signal (c.p.s.)	83	238	309	692
(b) Reaction time with antibody (min)	0	6	9	15
c.p.s.	3.5	38	43.6	47.8
Signal	—	32.5	38.1	42.3
(c) Phage (p.f.u.)	0	5×10^5	10^6	5×10^6
c.p.s.	22	35.5	49.1	146
Signal (c.p.s.)	—	13.5	27.1	124

A labelled mixed antibody reagent containing five components was used to assay *Brucella abortus*, *Escherichia coli*, *Pasteurella tularensis*, *P. pestis* or *Bacillus subtilis* spores with a reaction time of 6 min at 25 °C (Table 3). Results were based on equiv. bacterial dry wt rather than number because the formalized suspensions of pathogenic bacteria had aggregated and bacterial cell counts could not be determined; each of the bacterial species was detected and the radioactive signal given by a mixture of the bacteria was about 85 % of the total of the signals given by the same amounts of the individual bacterial species tested separately. Tests with other labelled mixtures of antibodies confirmed that their use for detection purposes was practicable but sensitivity was lower than that obtained with single labelled antibody reagents.

Indirect assay. Bacteria with attached rabbit homologous antibody take up ^{125}I -labelled anti-rabbit globulin and, therefore, detection of several bacterial species may be achieved with a single labelled antibody.

When an indirect assay was applied to samples of a suspension of killed *Pasteurella tularensis* (Schu 4), the assay blank value was high and the signal low (Table 4). Samples of suspensions of *Bacillus subtilis* spores and *Escherichia coli* were also tested by a number of modifications of the indirect radio-assay but the minimum number of bacteria detectable was 10^5 to 10^6 . Control tests showed that variable amounts of ^{125}I -labelled goat anti-rabbit globulin combined with all the bacteria (untreated with anti-bacterial antibody) tested; pre-adsorption of goat anti-rabbit antibody with the bacteria to be assayed decreased but did not necessarily eliminate uptake in the assay. Thus, the indirect bacterial assay is unreliable unless appropriate controls are included to check uptake of ^{125}I -labelled goat anti-rabbit globulin. The low signals may have been due to the relatively low specific immunological activity of the purified goat anti-rabbit globulin but products with similar activity were obtained using three types of immunoabsorbents (see Methods).

Table 8. *Detection of frozen and thawed phage T7 with ¹²⁵I-labelled anti-phage globulin*

(a) Samples (1 ml; 10^8 p.f.u.) phage T7 in water were frozen and thawed six times. Portions (0.1 ml) were incubated with ¹²⁵I-labelled anti-phage globulin reagent (0.05 ml; 0.5 µg antibody protein; 32 µCi/µg) at 30 °C for 6 to 60 min. Anti-phage serum (0.1 ml) was then added and incubation continued for 6 min.

(b) Portions (0.1 ml; initially 5×10^4 to 10^7 p.f.u.) of frozen and thawed phage suspension were incubated with double strength saline-phosphate buffer (0.1 ml) and ¹²⁵I-labelled antibody (0.05 ml; 0.5 µg antibody protein; 40 µCi/µg) for 15 min at 30 °C. Anti-phage serum (0.05 ml) was then added and incubation continued for 6 min.

Reaction mixtures were treated as described in Methods.

(a) Reaction time with antibody (min)	6	9	15	30	60		
Blank (c.p.s.)	6.5	5.0	7.7	10.4	10.5		
Signal (c.p.s.)	461	562	744	1014	1215		
(b) Initial phage p.f.u.		0	5×10^4	10^6	5×10^6	10^7	
c.p.s.		4.0	7.5	7.9	21	45	260
Signal (c.p.s.)		—	3.5	3.9	17	41	256

Detection of bacteria on the surface of a membrane filter

A method of detecting bacteria filtered on to a membrane filter was developed (see Methods) for assaying aqueous samples with a low bacterial content. A minimum of about 2×10^3 *Bacillus subtilis* var *niger* spores was detected after 'staining' with ¹²⁵I-labelled homologous antibody and the signal increased with the number of spores up to 10^4 (Table 5); about 10^4 *Pasteurella tularensis* or *Escherichia coli* on membrane filters were also detected. Results were reproducible, but the method was less accurate than the standard assay.

An indirect radio-antibody assay was also used to detect bacteria on membrane filters but sensitivity was low and the reproducibility of results poor.

Detection of bacteriophage T7

Before the radio-antibody assay could be applied to the detection of phage T7, a method of rapidly separating labelled phage immune complex had to be found. Membrane filters (Millipore VC and VM) of appropriate pore size were tested but filtration rates were slow and assay blank values high. Three tentative methods (see Methods) were devised but their application to the detection of other viruses has not been tested.

Phage attached to host bacterial cells. In the host cell method, labelled phage immune complex attached to *Escherichia coli* MRE160 was rapidly separated from excess radioactivity by filtration and washing on a Millipore membrane filter of 0.45 µm pore size. With a given number of phage, the signal increased with exposure time of phage to bacterial cells and labelled antibody (Table 6); the signal increased with phage concentration (Table 6) but the relationship was not linear.

Immunofiltration. The method depended on the fact that, after uptake of ¹²⁵I-labelled anti-phage globulin, phage particles still had sufficient free antigen sites for attachment to anti-phage globulin heat-fixed to a membrane filter but excess labelled antibody was washed through. Signals and assay blank values increased with the amount of antibody heat-fixed to membranes (Table 7) and the optimum amount was established by experiment. Increase in the reaction time of phage with labelled antibody up to 15 min increased the signal and, with a given reaction time, the relationship between phage concentration and signal was nearly linear (Table 7).

Freeze-thawed phage. The method depended on the fact that an immune complex of labelled antibody and phage damaged by freezing and thawing in distilled water was retained by a membrane filter (0.22 μ m pore size) even after extensive washing with saline-phosphate buffer + Brij 35. Presumably, freeze-thaw cycles increased the particle size and/or adherence of phage to a membrane filter. After six freeze-thaw cycles, the signal increased to 40-fold that given by untreated phage; the effect was greatest on phage in distilled water and decreased in the presence of salts or Brij 35. Increase in the reaction time of freeze-thawed phage with labelled antibody up to 60 min increased the signal and, with a given reaction time, the relationship between phage concentration and signal was nearly linear (Table 8).

Of the three assay procedures for phage T7, the freeze-thaw method gave the best results. About 10^5 p.f.u. (5×10^5 total particles; about 10^{-10} g equiv. dry wt) were unequivocally detected and sensitivity on a mass basis compared favourably with that achieved with the bacterial radio-antibody assays.

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