The Rapid Detection and Determination of Sparse Bacterial Populations with Radioactively Labelled Homologous Antibodies

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SUMMARY

An assay for rapidly detecting and determining sparse populations of vegetative bacteria or bacterial spores (minimum number about 500; 2 to 4 × 10^3 g. equivalent bacterial dry weight) is described. A sample is treated with ^125I-labelled purified homologous antibody, filtered and washed on a Millipore membrane filter, and the radioactivity of the separated labelled immune complex is measured. The assay is specific, accurate and completed within 8 to 10 min. Sensitivity and accuracy decrease if the assay is applied to samples that contain particulate matter that non-specifically attaches antibody and is retained by a membrane filter. This type of interference is decreased by pretreating samples with clarified normal rabbit serum for a few minutes before assay.

INTRODUCTION

Several solutions to the problem of detecting and determining small microbial populations in liquid samples within minutes have been proposed; instrumented techniques based on luminol chemiluminescence (Oleniacz, Pisano & Rosenfeld, 1966), staining (Nelson, Bolduan & Shurcliff, 1962) and other principles including firefly luminescence (Mitz, 1969) have been reported. The majority of the methods proposed have 'broad spectrum' capability, that is, the microbes present are detected but not specifically identified. Even if experience shows that the broad spectrum principles are sound and the sensitivities claimed are realistic, detection without identification is inadequate in a number of situations. For example, the microbial flora in natural waters vary qualitatively and quantitatively over wide limits, and analysis of samples with such methods would not show the presence of relatively small amounts of a specific micro-organism, e.g. an infective bacterium. Most of the methods reported for the rapid specific identification of microbes are based on immunofluorescence; since the invention of the technique (see Coons, Creech, Jones & Berliner, 1942), numerous applications are in routine use in microbiological and clinical diagnostic laboratories. In addition to fluorescent dyes as labels for antibody proteins, radioactive isotopes of iodine have been used (Hales & Randle, 1963; Miles & Hales, 1968) and an assay based on bacterial uptake of radioactively labelled antibody should be extremely sensitive. In practice, however, determination of a few bacteria is possible only if, after separation of the labelled-immune complex, the variation in the level of non-specifically attached radioactivity is less than the radioactivity of the immune complex. If residual non-specifically attached radioactivity (determined by testing 'blank' samples without bacteria) is high compared with the radioactivity of the immune complex, even relatively small variations will swamp the signal from the complex. High erratic assay blank values were a major problem that has now been largely resolved.

We now describe an assay based on the use of ^125I-labelled antibodies for the rapid specific detection and determination of small numbers of bacteria in aqueous suspension.
Bacteria. Bacillus subtilis var. niger spores were provided as a thick suspension (3 to 6 x 10^10 spores/ml.) by Microbial Products Section, Medical Research Establishment (MRE). The crude spores were purified with the method of Sacks & Alderton (1961). Strains of Escherichia coli were grown in shaken flasks containing nutrient broth for 17 h. at 37°, harvested and washed in saline phosphate buffer (0.11 M-NaCl containing 0.02 M-sodium phosphate buffer, pH 7.7) by centrifugation and resuspended (about 10^10 viable bacteria/ml.) in saline phosphate. Bacterial suspensions were assessed with viable and total counts, and dry-weight determinations (bacteria washed with distilled water and dried at 103° for 16 h.).

Purification of antibodies. High titre antisera against Bacillus subtilis spores and Escherichia coli cells were produced in rabbits and sheep, respectively. Purified B. subtilis spores (10^9/ml.) were injected intravenously into rabbits (8 doses of 1 ml. during 4 weeks) and blood was collected after 5 weeks. Sheep received 4 doses of 5 ml. of washed viable E. coli cells (10^9/ml.) over 40 days and were bled after 50 days. Serum was separated by centrifugation, filtered through a Millipore membrane filter (GSWP: 0.22 μm.) and stored at -15°.

125I-Labelled antiserum or a salt-precipitated globulin fraction from it was not suitable for determining less than 10^5 to 10^6 bacteria. When crude antisera are labelled, the iodine attaches to the extraneous protein as well as to the antibody itself; this increases the amount of radioactivity non-specifically adsorbed relative to that associated with the antibody and attached to the homologous bacteria. Therefore, immuno-purified antibody was prepared to attain higher sensitivity. The method used was based on one developed previously by our colleagues Dr B. T. Tozer and Dr A. P. MacLennan (unpublished work).

Antibody was adsorbed from antiserum (20 ml.) with washed homologous bacteria (200 to 500 mg. equiv. dry wt) for 2 h. at 37° and 24 to 48 h. at 2°. Anti-Bacillus subtilis spore serum was adsorbed with purified viable spores and anti-E. coli MRE 162 serum with stationary phase E. coli MRE 162 or related serotype (see below) that had been boiled in 0.85 % NaCl under reflux for 2 h. and then thoroughly washed with 0.85 % NaCl by centrifugation. After adsorption, the resulting immune complex was separated and washed three times with 0.85 % NaCl by centrifugation and dissociated with a suitable agent. Several dissociating agents were investigated and the best for use with spore immune complex was 3.5 M-KCNS (see Dandliker & DeSaussure, 1969) but this agent was unsuitable for use with vegetative cell immune complexes because the bacteria themselves partially dissolved. Escherichia coli immune complexes were dissociated with 0.37 M-glycine-HCl buffer (pH 2.5) or 1 % (w/v) NaCl-0.1 N-HCl buffer (pH 1.2). Washed immune complex was dispersed in ice-cold dissociating agent (15 ml.) and the suspension gently stirred for 15 min. at 2°. Soluble antibody was separated by centrifugation and the residue was re-extracted with dissociating agent (5 ml.). The combined glycine buffer or thiocyanate extracts were filtered through a Millipore membrane filter (GSWP; 0.22 μm. pore size) and either neutralized or diluted and dialysed against distilled water respectively. The antibody solution was concentrated to about 1 ml. by pressure dialysis through cellophane at 2° and insoluble material removed by centrifugation. Further purification of the eluted protein was usually necessary to provide a product suitable for use in the assay.

In earlier work, IgM was separated by fractionating the eluted antibody on a column of Sephadex G 200 (Pharmacia, Uppsala, Sweden) and IgM was discarded. Later it was found that satisfactory material was obtained by fractional precipitation of eluted antibody with (NH₄)₂SO₄. Two fractions precipitating at 0 to 33 % and 33 to 50 % saturation of salt,
respectively, were separated by centrifugation, redissolved in saline phosphate buffer (pH 7.7) and dialysed against this buffer until \( \text{NH}_4^+ \) was no longer detected. Both fractions had high specific immunological activity but generally the second fraction was the more suitable for assay purposes. Final yield of purified antibody depended on the antiserum, dissociating agent and extent of spontaneous precipitation that occurred during processing. In the case of anti-\textit{Bacillus subtilis} spore globulin (33 to 50% saturated \((\text{NH}_4)_2\text{SO}_4\) fraction of eluted protein) about 2 to 4 mg. protein was obtained from 20 ml. antiserum (agglutination titre with spores, \(1:1000\) to \(2000\)).

Immuno-purified globulins prepared from antiserum against \textit{Escherichia coli} MRE 162 (O8 K9) contained antibodies against O8 and K9 antigens; purified antibodies specific for each of these antigens were obtained by adsorbing the antiserum successively with \textit{E. coli} serotypes O8 K8 and O9 K9, respectively, and dissociating the immune complexes (A. P. MacLennan, unpublished work).

**Iodination of anti-bacterial globulins.** The method was essentially as described by Hunter & Greenwood (1962) and Haber, Page & Richards (1965) for the iodination of small amounts of polypeptide hormones. Antibody protein (25 to 250 \(\mu\)g. in 5 to 50 \(\mu\)l. saline buffer, pH 7.7), 2 to 3 mCi Na\(^{125}\)I (carrier-free, free from reducing agent; from The Radiochemical Centre, Amersham, Buckinghamshire) and chloramine T (0.01 M in saline buffer, 20 \(\mu\)l.) were mixed by injection from a micro-syringe into a small tube (7.6 \(\times\) 0.8 cm.). After 3 min. at \(20^\circ\), sodium metabisulphite (0.01 M; 20 \(\mu\)l.), potassium iodide (10 mg./ml.; 40 \(\mu\)l.) and clarified normal rabbit serum (0.1 ml.) were added in the order given; the mixture and saline phosphate washings (0.5 ml.) were filtered through a column (30 \(\times\) 1.2 cm.) of Sephadex G25 (Pharmacia, Uppsala, Sweden) with saline buffer containing 0.1% Brij 35 (polyoxyethylene lauryl ether from British Drug Houses Ltd, Poole, Dorset) as the eluting solution. Excluded protein was collected in 4 to 6 fractions (0.5 ml.) that were combined, mixed with more clarified normal rabbit serum (\(1/4\) final volume) and diluted to 5 to 7.5 ml. with saline buffer. The radioactivities of the antibody solution, and the combined fractions of small molecular weight material that emerged from the column later, were measured and the uptake of \(^{125}\)I by antibody protein determined. Usually 80% or more of the radioactivity was taken up and the specific radioactivity of the antibody varied between 6 and 50 \(\mu\)Ci/\(\mu\)g. protein. Labelled antibody reagents were stored at \(2^\circ\) and had a useful life of 3 to 30 days depending on the level of labelling.

**Assay procedure.** All solutions were filtered through Millipore membrane (GSWP; 0.22 \(\mu\)m.) immediately before use. Samples (0.1 to 1 ml.) of bacterial suspension and saline buffer alone in small tubes (10 \(\times\) 1 cm.) were mixed with labelled antibody reagent (0.05 to 0.1 ml.) delivered from a hypodermic syringe attached to a Millipore Swinny filter holder (13 mm. diam.) fitted with a Millipore membrane (0.22 \(\mu\)m.). The mixtures were held at 20 to 25\(^\circ\) for the reaction time selected, diluted with saline buffer containing 1% Brij 35 (1 to 2 ml.) and rapidly filtered by suction through discs (\(2\) in. diam.) of Millipore membrane (HAWP, 0.45 \(\mu\)m.) lying on a disc (\(3/4\) in. diam.) of Whatman no. 1 paper held in the filter jigs (see below). The filter was rapidly washed with saline buffer + 1% Brij 35 (3 \(\times\) 1 ml.) and the top of the filter jig removed. The entire surface of each membrane filter was then washed for at least 1 min. with a drip-feed of saline buffer + 1% Brij 35. Each washed membrane filter was placed between discs (\(3/4\) in. diam.) of linen tracing paper and a central area slightly less (8%) than the filtration area was accurately cut out with a compound blanking punch (see below), and collected into a glass vial (3.5 \(\times\) 1 cm.). All samples and blanks were tested in duplicate and the signal for a given number of bacteria was determined by subtracting the mean blank counts from the mean test counts.
The filter jig and press

The construction of the jig is shown in isometric projection (quarter section) in Fig. 1. It is made of stainless steel for the sake of robustness, which conduces to safety in handling radioactive and pathogenic material.

![Diagram of the filter jig](image)

**Fig. 1.** Filter jig: isometric projection, quarter section. A, Gauze or sintered stainless steel plate; B, O-ring; C, locating pins; D, hole communicating with bore of tail-pipe.

The tail-pipe of the lower member of the jig is connected by way of a tap with a vacuum manifold; the manifold is provided with a reservoir for liquid passing the filter and a column of iron turnings to trap corrosive vapours (e.g. I₂). The filter material itself—a disc of Millipore membrane ¼ in. in diameter—is supported by a fine stainless steel gauze or a sintered plug let into the lumen of the tail pipe (A, Fig. 1), and centred by three pins (C). These pins also mate with holes in the upper member of the jig to ensure the concentricity of the whole assembly. The seal between the two members is formed by a soft O-ring (B), which in the unstressed state is about 0.01 in. proud of the adjacent upper steel surface. The tail-pipe communicates with the annular gap between the two members by way of a port (D). Thus when a vacuum is applied the two are urged together by a force equivalent to about 20 kg.;
the O-ring gives easily, so that most of this force is applied to grip the filter membrane. (In a later design the port (D) can be closed by a needle valve.)

After a sample has been filtered and washed, and the vacuum turned off, the upper part of the jig is easily removed by a vertical pull; it is important to avoid bruising its lower polished surface by contact with the pins (C) or other hard edges.

Although the particulate matter on the filter is confined to the area over the lumen of the tail-pipe, there is lateral seepage of liquid through the body of the membrane, in spite of the force with which it has been compressed. This seepage carries towards the edge of the membrane soluble radioactive matter which cannot be removed by washing in situ. Accordingly the filter membrane is placed between two similar discs of smooth paper or plastic material and the centre (½ in. diam.) punched out in a small blanking press; the plastic prevents contamination of the punch and die.

The die of the press (⅛ in. o.d.) is sunk in the bolster, and the punch is surrounded by a spring-loaded sleeve (also ⅛ in. o.d.). When the press is operated the sleeve first meets the filter membrane and holds it flat; further pressure then advances the punch. The punchings are received in a small tube held under the hole in the die.

Measurement of radioactivity. The radioactivity of membrane discs was measured with a conventional well-type sodium iodide scintillation counter and scaler with a counting efficiency for $^{125}\text{I}$ of about 40%; instrument background counts were 50 to 100 c.p.m. Counts were usually collected for 1 min.

Determination of protein. Ultraviolet measurements at 260 and 280 nm. (Warburg & Christian, 1941) and the method of Lowry, Rosebrough, Farr & Randall (1951) were used with dried bovine $\gamma$-globulin (Cohn fraction I from Sigma Chemical Company) as the standard.

RESULTS

Factors affecting the sensitivity and reproducibility of the assay. These factors were the quality, reaction concentration and specific radioactivity of the $^{125}\text{I}$-labelled antibody and, in particular, the level and reproducibility of the blank value in the absence of bacteria. Purified antibodies suitable for the assay showed high specific and low non-specific binding activity, assayed as pure proteins and produced one band in the position of $\gamma$-globulin on electrophoresis on a cellulose acetate strip at pH 8.6; preparations containing IgM were generally less suitable than pure IgG preparations because they gave rise to high and variable blank values. Many types of membrane filter were tested for assay purposes and Millipore mixed cellulose ester membranes were among those giving the lowest blank values. Non-specific binding of labelled antibody was decreased to a low and fairly reproducible level by including normal serum in the antibody reagent, adding the minimum amount of reagent commensurate with sufficient uptake of antibody during the reaction time allowed and washing the membrane with 1% Brij 35 in saline buffer to remove excess radioactivity.

The minimum amount of labelled antibody required to achieve sufficient uptake for readout purposes depended upon the number of bacteria present, the sample volume and the reaction time. At least 3 μg. of labelled antibody was needed to obtain a sufficient uptake by less than about $5 \times 10^3$ bacteria/ml. of sample within a few minutes. At this concentration, antibody was in large excess until the number of bacteria or spores exceeded $10^6$/ml. (bacterial dry wt, 0.3 to 0.7 μg./ml.) and a lower concentration sufficed with a longer reaction time. Fig. 2 shows uptake by $5 \times 10^3$ Bacillus subtilis spores in the presence of 3 μg. $^{125}\text{I}$-labelled antibody/ml. during 60 min. at 25°.

With less than $5 \times 10^3$ bacteria in the sample tested, the accuracy of the assay improved
markedly when the sample volume was decreased from 1 to 0.1 ml. A suitable antibody concentration was provided with the addition of less reagent resulting in a proportional fall in the blank value and its experimental variation. For example, radioactive material adsorbed on to the membrane filter when 0.1 and 1 ml. of saline buffer containing 3.3 μg 125I-labelled anti-Bacillus subtilis spore globulin (8 μCi/μg.)/ml. were filtered and washed as described above gave 45 and 260 c.p.m., respectively. If the number of bacteria in 0.1 ml. of sample is too small to be determined, either a larger sample can be tested or the bacteria concentrated into a smaller volume. Dilute bacterial suspensions were rapidly concentrated by filtering a stirred sample (10 ml.) through a washed Millipore membrane filter (0.45 μm.) in a Pyrex microanalysis filter holder (25 mm.) under reduced pressure until the volume fell to 1 ml. The stirrer vanes were positioned to rotate slightly above the membrane surface and the motor speed was adjusted to produce a vortex extending to just above the vanes. Good recoveries of Escherichia coli cells and B. subtilis spores were obtained with stirring whereas without it losses were high because of attachment of the organisms to the filter membrane.

The sensitivity of the assay depended on the specific radioactivity of the 125I-labelled antibody. When samples containing the same number of bacteria were reacted for 5 to 6 min with the same concentrations of 125I-labelled antibody of increasing specific radioactivity, radioactive signals given by the separated immune complexes increased with specific radioactivity of the protein up to at least 4 atoms 125I/molecule (assuming a molecular weight of
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Fig. 3. Relationship between number of bacteria and the radioactive signal given by the separated immune complex. Samples (0.1 ml.) of suspensions of washed viable Escherichia coli (K99g H12) cells (O) and purified Bacillus subtilis spores (●) were reacted with $^{125}$I-labelled anti-K9 globulin (50 μl.; 0.6 μg. antibody protein; 8 μCi/μg.) and anti-B. subtilis spore globulin (50 μl.; 0.5 μg. antibody protein; 33 μCi/μg.) respectively, for 6 min. at 25°. Reaction mixtures were processed and radioactivities measured as described in the text. The mean radioactive blank levels were 96 and 48 c.p.m. with antibodies to E. coli cells and B. subtilis spores, respectively.

Table 1. Effect of radioactive concentration of $^{125}$I in antibody protein on the signal given by $10^4$ purified Bacillus subtilis spores

Samples (0.1 ml.) of spore suspension and saline buffer were treated with $^{125}$I-labelled anti-B. subtilis spore globulin (50 μl.; 0.25 μg. antibody protein; 0.74 to 4.1 atoms $^{125}$I/molecule antibody) for 6 min. at 25°. Reaction mixtures were processed as described in the text.

<table>
<thead>
<tr>
<th>Atoms $^{125}$I Molecule antibody protein</th>
<th>c.p.m.</th>
<th>Signal</th>
<th>Blank</th>
<th>Signal Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.74</td>
<td>16</td>
<td>384</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>1.11</td>
<td>22</td>
<td>681</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>1.49</td>
<td>37</td>
<td>1052</td>
<td>28</td>
<td></td>
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<tr>
<td>2.60</td>
<td>85</td>
<td>1376</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>4.10</td>
<td>116</td>
<td>2122</td>
<td>18</td>
<td></td>
</tr>
</tbody>
</table>
160,000). Since, however, the blank value and its experimental variation also increased, there was no practical advantage in labelling antibody with more than 2 to 3 atoms $^{125}$I/molecule protein (Table 1). A further disadvantage of more highly labelled antibodies was their inferior storage properties; this was presumably due to an increase in radiation-induced damage.

**Assay of Bacillus subtilis spores and Escherichia coli cells.** The relationship between signal and number of washed *Escherichia coli* (O9 K9) cells and Bacillus subtilis spores, each reacted for 6 min. at 25° with $^{125}$I-labelled homologous antibody, is shown in Fig. 3. The signal was directly proportional to the number of bacteria from about 500 to $10^5/0.1$ ml. sample and a similar relationship held with most of the batches of purified antibody tested. Occasionally, the signal per bacterium decreased progressively as the number of bacteria in the sample was increased; this was presumed to be due to the immunological heterogeneity of antibody molecules present in the preparation. The sensitivity of the assay for *E. coli* MRE162 (O8 K9) cells with $^{125}$I-labelled anti-K9 globulin was slightly lower than for *E. coli* serotype O9 K9 but a minimum of 1000 cells was reproducibly detected.

The signal with samples of *Bacillus subtilis* spores depended on the purity of the spore suspension; samples of diluted crude spore suspension gave two to three times the signal given by samples of purified spore suspension containing an equivalent number of intact spores. The high signal with unpurified spores was apparently due to the presence of non-viable immature spores in the stock suspensions not scored in viable and total counts and/or to spore debris containing spore antigens. Labelled purified immune globulins against several other bacteria were not taken up by purified or unpurified spores; vegetative cell debris was present in the crude spore suspensions but intact vegetative *B. subtilis* did not take up labelled anti-spore globulin.

No appreciable uptake of antibody occurred when $10^8$ *Bacillus subtilis* spores or *Escherichia coli* MRE162 were treated with $^{125}$I-labelled anti-*E. coli* K9 globulin and anti-*B. subtilis* globulin, respectively. Neither labelled antibody was taken up to a significant extent by *E. coli* serotype O55B5H6 or *Aerobacter aerogenes* NCTC 418 cells.

The results given refer to washed suspensions of bacteria; sensitivity of the assay is decreased if samples contain an excess of particulate matter that non-specifically attaches antibody and is retained by a membrane filter. This type of interference is often markedly decreased if the sample is treated with Millipore membrane-filtered normal rabbit serum (0.1 to 0.2 ml./ml. sample) for a few minutes before assay with radioactively labelled antibody.

**COMMENT**

The assay described is specific, accurate and completed within 8 to 10 min. The sensitivity obtained depends on the time allowed for labelled antibody to react with the bacteria but, under the conditions described, 5 to 6 min. was sufficient to allow detection of 500 to 1000 vegetative bacteria or bacterial spores. The reaction time can be decreased if larger numbers of bacteria are present in samples. We have not reached the theoretical sensitivity of the assay because experimental variations in the test and blank responses were sufficient to swamp the signal given with less than about 500 bacteria.

The disadvantage of the specific immunological assay is that it allows of detecting and estimating only one species of organism at a time. We have shown that a radioactively-labelled mixture of several type-specific antibodies can be used to determine any one of the homologous bacteria but sensitivity is lower than with a single labelled antibody.
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REFERENCES


