Comparative Study of $^{14}$C-Labeled Purified Protein Derivative from Various Mycobacteria

I. Preparation of $^{14}$C-Labeled Purified Protein Derivative Antigens and Their Adsorption to Glass

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Received for publication 27 May 1970

Biologically active $^{14}$C-labeled purified protein derivative ($^{14}$C-PPD) has been prepared from the culture filtrates of seven species of mycobacteria, namely *Mycobacterium tuberculosis* Johnston strain (PPD), *M. bovis* BCG (PPD-BCG), *M. avium* (PPD-A), *M. kansasii* (PPD-Y), *M. intracellulare* (PPD-B), *M. scrofulaceum* (PPD-G), and *M. fortuitum* (PPD-F). These mycobacteria were grown in a culture medium containing a mixture of $^{14}$C-labeled amino acids. The yield and specific radioactivity of the PPD, of the nucleic acid, of the bacterial cells, and of the CO$_2$ developed during growth have been determined for each of the seven species of mycobacteria. Although the yields of $^{14}$C-PPD antigens differed greatly for the different species of mycobacteria tested, their specific radioactivities were similar. The $^{14}$C-PPD antigens have been used as a means to measure their adsorption to glass. When glass ampoules containing dilute solutions (0.001 mg of PPD per ml) of these PPD antigens (PPD, PPD-BCG, PPD-A, PPD-Y, PPD-G, PPD-B, and PPD-F) were stored for 12 months at 5°C, it was found that they all adsorbed equally well to glass surfaces. In fact, regardless of the origin of the PPD, a loss due to adsorption of about 90% occurred during the first month of storage, and thereafter the PPD content remained practically constant for the rest of the duration of the storage period. The addition of 0.0005% Tween 80 to the PPD solutions effectively reduced the adsorption to glass of most PPD antigens. However, adsorption of PPD-BCG was not quite so effectively prevented, even when the Tween 80 concentration was increased from 0.0005 to 0.005%.

Preparations of $^{14}$C-labeled tuberculin purified protein derivative (PPD) obtained from the culture filtrate of *Mycobacterium tuberculosis* Johnston strain have been reported previously by Landi et al. (5) and have been used to measure the adsorption of tuberculin PPD to glass and plastic surfaces (4). These preparations have also been used as a screening method to find out what substance could be added to a dilute tuberculin PPD solution to prevent the adsorption of tuberculoprotein to the wall of the container. To extend these adsorption studies to PPD obtained from other species of mycobacteria, in addition to $^{14}$C-labeled tuberculin PPD obtained from *M. tuberculosis* Johnston strain, $^{14}$C-labeled PPD antigens were also prepared from *M. bovis* BCG (PPD-BGG), *M. avium* (PPD-A), *M. kansasii* (PPD-Y), *M. scrofulaceum* (PPD-G), *M. intracellulare* (PPD-B), and *M. fortuitum* (PPD-F). [The designation of atypical, unclassified, or anonymous mycobacteria for these species, as referred to by many investigators, has been avoided in this article and the nomenclature used is that recommended by Runyon (10) and Wayne et al. (14).] The present paper describes in detail the preparation of these various radioactively labeled PPD antigens and compares the yields and specific radioactivities of these PPD antigens and of the bacterial cells and CO$_2$ developed during growth between the various species of mycobacteria used. Adsorption studies were carried out with these $^{14}$C-PPD preparations to find out, first, if these PPD antigens would adsorb equally well to the glass wall of the containers and, second, if Tween 80 at the concentration of 0.0005% (5 μg/ml) as used in tuberculin PPD solutions (4) would also prevent adsorption of these PPD antigens to glass.
MATERIALS AND METHODS

Reagents. Phenol (analytical reagent grade) was supplied by the British Drug Houses Ltd., Poole, England; Tween 80 [polyoxyethylene (20) sorbitan mono-oleate] was supplied by Atlas Chemical Industries, Wilmington, Del.

Phosphate-buffered saline, pH 7.38. Isotonic phosphate-buffered saline (2, 8), containing 1.45 g of KH₂PO₄, 7.60 g of Na₂HPO₄·2H₂O, and 4.80 g of NaCl per liter, was used. Phenol (0.3%) was added as a preservative.

Cultures. The stock cultures of the organisms were maintained on Loewenstein medium by bimonthly subculture. The seed cultures were maintained by serial passages every 10 days on Long's synthetic medium (7). However, M. bovis BCG was subcultured alternately on glycerinated water-potato, on glycerinated bile-potato, and on Sauton medium (9). The source of cultures and designation of the ¹⁴C-PPD antigens are listed in Table 1.

Medium and seeding. Long's medium (7) was used for the production of ¹⁴C-PPD. One liter of medium was dispensed into a 5,000-ml Povitzky bottle. The bottle of medium was sterilized in the autoclave by 10 min of steaming, followed by 30 min of 15 psi of pressure and 121 C. After cooling, a mixture of 15 purified ¹⁴C-labeled L-amino acids (1 mCi), obtained from New England Nuclear Corp. (catalogue no. NEC-445) and sterilized by filtration through a Gelman filter (pore size, 0.45 μm), was added to the medium.

The organism under investigation was grown at 37°C in a 250-ml Erlenmeyer flask on Long's synthetic medium as a surface pellicle for approximately 10 days. Some of this pellicle was then inoculated into a Povitzky bottle containing the radioactive medium and was incubated at 37°C for 6 to 17 weeks under controlled aeration, depending on the rate of growth of the organism under investigation.

Aeration and carbon dioxide trapping. The Povitzky bottle was closed with a rubber stopper containing a long glass tube for air inlet (extending to the middle of the bottle) and a short glass tube for air outlet (extending only about 1 cm into the bottle). Each tube was connected to air filters (tubes containing sterile cotton). The air-filtration system from the outlet tube was connected with rubber tubing to two CO₂ traps placed in series and containing 2 N NaOH. These traps were connected via an airflow meter and a pressure regulator to a water jet aspirator pump (Fig. 1). An identical airflow system was employed for the Povitzky bottle (Fig. 1) used as a control. The aeration was kept at a rate of 400 ml/hr (= 9.6 liter/day) as measured at the outlet. The amount of CO₂ developed was determined at intervals (usually 2 to 3 days) by potentiometric titration and radioactive assay of samples of the CO₂-trapping solution.

Bacterial cell preparations. The culture bottle was steamed for 3 hr (100°C) in an autoclave. The bottle was allowed to stand overnight at room temperature. The main part of the growth was removed from the medium by centrifugation (1,400 × g), then washed twice with 200 ml of distilled water, and dried in a vacuum desiccator over CaCl₂.

Culture filtrate. The supernatant from the centrifugation was filtered through Whatman no. 1 paper to remove some residual cells. The filtrate obtained was then a clear amber solution.

¹⁴C-PPD, trichloroacetic acid precipitate. A 44% trichloroacetic acid solution was added to the culture filtrate to give a final concentration of 4%. The total protein was precipitated and centrifuged at 1,400 × g for 20 min. The precipitate was redissolved in 40 ml of 0.07 M NaHPO₄, resulting in a slightly turbid solution. This solution was clarified by high-speed centrifugation (30,900 × g for 4 hr) in a refrigerated centrifuge (4°C), and the deposit ("insoluble matter in the first trichloroacetic acid-precipitate") was vacuum-dried over CaCl₂ and NaOH. Trichloroacetic acid was added (4% final concentration), to the clear
supernatant and the precipitate obtained was vacuum-dried over CaCl₂ and NaOH. Unless otherwise stated, this second trichloroacetic acid precipitate will be referred to throughout this work as "trichloroacetic acid precipitate."\(^9\)

\(^{14}\)C-PPD, ammonium sulfate precipitate. In some cases, the trichloroacetic acid precipitate was further purified by redissolving it in 0.07 M Na₂HPO₄ to form a 2% solution. To this solution an equal volume of neutralized saturated ammonium sulfate (pH 7) was added. The precipitate obtained was washed free from ammonium sulfate by repeatedly (ca. six times) stirring with 4% trichloroacetic acid solution and centrifuging, until the supernatant was free from ammonium sulfate. It was then washed with acetone (two times) and ether and vacuum-dried over CaCl₂.

\(^{14}\)C-PPD stock solutions. All radioactive PPD preparations were dissolved in 0.033 M Na₂HPO₄ to form solutions containing 0.5% \(^{14}\)C-PPD. These solutions were sterilized by filtration through a Gelman filter (pore size, 0.45 μm) and stored at approximately -20°C.

Preparation and dispensing of \(^{14}\)C-PPD solutions. Dilute solutions of \(^{14}\)C-tuberculin PPD, with and without Tween 80, were prepared in phosphate-buffered saline (pH 7.38) containing 0.3% phenol and 0.001 mg of tuberculin \(^{14}\)C-PPD (50 tuberculin units per ml) per ml. [One tuberculin unit equals 0.00002 mg of the international standard PPD (PPD-S; 1).] These solutions were dispensed into 10-ml glass ampoules in portions of 2.5 ml. The ampoules were stored at 5°C and withdrawn after various times to be assayed for their radioactivity in solution.

Solutions of \(^{14}\)C-PPD antigens obtained from the culture filtrates of six species of mycobacteria other than \(M.\) tuberculosis were prepared and dispensed in an identical manner as described for \(^{14}\)C-tuberculin PPD.

Ampoules. Glass ampoules of known composition \((4)\) from Owens-Illinois Inter-America Corp., Toledo, Ohio, were used (ampoule designation, N51A break 120/12 fl. 2). Closure was effected by flame-sealing of the ampoules which had an inner glass surface of 24.2 cm².

Spectrophotometry. Ultraviolet spectra of the various PPD antigens were taken with a Beckman DU spectrophotometer. The PPD obtained from mycobacteria contains two ultraviolet-absorbing substances, protein and nucleic acid. Quantitative determinations of the nucleic acid were made after separating it from the protein by paper electrophoresis and expressing it in per cent of weight and of the radioactivity of the PPD. The paper electrophoresis method used had been described previously in 1965 by Landi and Held \((3)\).

Radioactivity determinations. \(^{14}\)C-labeled material was assayed in quadruplicate by evaporating portions of the liquid sample to dryness on stainless-steel ringed planchets \((31 \text{ mm in diameter}; \text{ Planchets Inc., Chelsea, Mich.})\) which were then counted in a gas-flow Geiger-Müller counter fitted with a Micromil window \((\text{Nuclear-Chicago Corp.})\).

To determine the distribution of both the weight and the radioactivity between protein and nucleic acid, the PPD antigens were separated by paper electrophoresis into these two groups of compounds. The nucleic acid was extracted and assayed quantitatively by ultraviolet spectrophotometry and by radioactivity count. The difference in weight between the original PPD preparation and the nucleic acid (as determined by ultraviolet spectrophotometry) was taken as the weight of the protein. The difference in radioactivity between the original PPD preparation and the nucleic acid (as determined by \(^{14}\)C assay) was taken as the radioactivity of the protein. From these data, the specific radioactivities of protein and nucleic acid were calculated.

The radioactivity of all insoluble material was determined after wet combustion with a mixture of \(\text{H}_2\text{SO}_4, \text{H}_3\text{PO}_4, \text{CrO}_3,\) and \(\text{KIO}_3\) \((12)\) whereby the \(^{14}\)CO₂ was trapped in 1 N NaOH solution. The NaOH...
containing \( \text{^{14}CO}_{2} \) was counted as described for the liquid samples.

In the adsorption studies, two ampoules were withdrawn after various times of storage, and samples of 1 ml were plated in quadruplicate and counted as described for the liquid samples. The results are expressed directly in percentage radioactivity in solution, the original radioactivity in solution (at zero time) was taken as 100% and the coefficient of variation (11) was 3.54%. When 1 ml of a 0.001 mg/ml solution of \( \text{^{14}C-PPD} \) in phosphate-buffered saline was plated, a radioactivity of approximately 110 counts/min above background was measured. The loss of radioactivity in \( \text{^{14}C-PPD} \) solution was attributed to adsorption of \( \text{^{14}C-PPD} \) on glass, since for \( \text{^{14}C-tuberculin} \) PPD the lost radioactivity was found on the inner glass surface (4).

**Skin tests in guinea pigs.** Comparisons of the potency of the various \( \text{^{14}C-PPD} \) antigens were made with solutions of equal concentration of the corresponding nonradioactive PPD. Samples of the radioactive and of the corresponding nonradioactive PPD stock solutions were diluted in phosphate-buffered saline containing 0.3% phenol and 0.0005% Tween 80 to give concentrations of 0.004 and 0.001 mg/ml, respectively. Doses of 0.1 ml were injected intradermally into a total of 56 white female guinea pigs, sensitized in seven groups of 8 guinea pigs with the corresponding species of heat-killed mycobacteria. The sensitizing injection was given intramuscularly in the inner part of the thigh. The dose injected in all animals was 0.1 ml of a 10 mg/ml amount of the appropriate heat-killed bacilli suspension in Freund’s adjuvant and saline mixed in equal proportions. The flanks of each guinea pig were divided into four squares. Two dose levels of the radioactive PPD and nonradioactive PPD were assigned in duplicate to the eight positions on each animal in a random fashion. At 24 hr after the injections, the longitudinal and transverse diameters of each reaction zone were measured in millimeters, and the result was recorded as the sum of these two diameters. The estimate of potency was made by the method of Long, Miles, and Perry (6).

**RESULTS AND DISCUSSION**

**Rate of development of CO\(_2\).** Figure 2 shows the rate of release of \( \text{^{14}CO}_{2} \) during the growth of seven species of mycobacteria used for the preparation of \( \text{^{14}C-PPD} \). \( \text{^{14}C-PPD-BCG} \), \( \text{^{14}C-PPD-A} \), \( \text{^{14}C-PPD-Y} \), \( \text{^{14}C-PPD-G} \), \( \text{^{14}C-PPD-B} \), and \( \text{^{14}C-PPD-F} \). The maximum rate of \( \text{^{14}CO}_{2} \) developed under our experimental conditions was parallel to the maximum bacterial growth as estimated from the daily visual observation of the culture. The maximum rate of nonradioactive carbon dioxide released was reached a few days after the maximum rate of \( \text{^{14}CO}_{2} \) had been reached, indicating that the mixture of \( \text{^{14}C} \)-amino acids had been metabolized earlier than the mixture of the nonradioactive organic substances present in the medium.

**Yield of PPD.** Table 2 shows the yield of PPD from the culture filtrates of the seven species of mycobacteria as well as the yield of cells, the amount of insoluble trichloroacetic acid precipitated matter obtained, and the amount of \( \text{CO}_{2} \) released during the growth period. The yield of PPD obtained from \( \text{M. tuberculosis}, \text{M. kansasi}, \) and \( \text{M. bovis} \) was much greater than the yield of PPD obtained from mycobacteria such as \( \text{M. avium}, \text{M. scrofulaceum}, \text{M. intracellulare}, \) and \( \text{M. fortuitum} \).

**Recovery of radioactivity.** Most of the radioactivity was present in the bacterial cells (Table 3). A considerable amount (6 to 14%) of the radioactivity was recovered as carbon dioxide, whereas 1 to 22% of the original radioactivity was incorporated in the PPD (trichloroacetic acid precipitate) as follows: from \( \text{M. tuberculosis}, 22.12\%; \text{M. bovis}, 9.60\%; \text{M. avium}, 3.90\%; \text{M. kansasi}, 7.60\%; \text{M. scrofulaceum}, 1.38\%; \text{M. intracellulare}, 5.36\%; \text{M. fortuitum}, 1.19\%.

Table 4 shows the specific radioactivity of the preparations. When a mixture of \( \text{^{14}C} \)-labeled amino acids was used, the specific radioactivity of \( \text{^{14}C} \)-protein was the highest among the substances formed during bacterial growth. In fact, the bacterial cells were approximately 21 to 61% as radioactive, the nucleic acid was 11 to 38% as radioactive, and the carbon dioxide was 2 to 5% as radioactive as the protein. The specific activity of the PPD antigens so obtained was on the order of 0.25 \( \mu \text{Ci}/\text{mg} \) (Table 4, footnote a).
of radioactivity is high enough to perform adsorption studies (4) and yet is very low when compared to the original specific activity of the amino acid mixture (ca. 1,400 μCi/mg) added to the medium. However, since only 0.7 mg (1,000 μCi) of amino acids was added to the medium, it would seem possible, if it were necessary, to produce preparations of much higher specific specific activity.

### Table 2. Yield of PPD, bacterial cells, CO₂, and insoluble matter obtained during the growth of various mycobacteria

| Determination                  | M. tuberculosis | M. bovis BCG | M. avium | M. kansasii | M. scrofulaceum | M. intracellulare | M. fortuitum |
|-------------------------------|-----------------|--------------|----------|-------------|-----------------|------------------|--------------|
| Cells                         | 4.01            | 5.04         | 2.69     | 8.66        | 1.85            | 7.58             | 6.72         |
| Carbon dioxide                | 8.72            | 10.38        | 7.10     | 6.63        | 8.00            | 7.92             | 18.52        |
| Insoluble matter in the first trichloroacetic acid precipitate | 0.585 | 0.051 | 0.007 | 0.032 | 0.025           | 0.063            | 0.000        |
| PPD (trichloroacetic acid precipitate) | 0.835 | 0.353 | 0.133 | 0.395 | 0.028           | 0.196            | 0.054        |
| PPD (ammonium sulfate precipitate) | 0.482 | NP | NP | 0.138 | NP             | 0.069            | NP           |

*a* All yields are expressed in grams per liter of culture medium.  
*b* Not prepared.

### Table 3. Recovery of radioactivity (¹⁴C) during the growth of various mycobacteria

| Determination                  | M. tuberculosis | M. bovis BCG | M. avium | M. kansasii | M. scrofulaceum | M. intracellulare | M. fortuitum |
|-------------------------------|-----------------|--------------|----------|-------------|-----------------|------------------|--------------|
| Cells                         | 35.30¹          | 61.38        | 48.40    | 42.60       | 46.78           | 62.90            | 56.81        |
| Carbon dioxide                | 11.86           | 13.71        | 9.22     | 7.28        | 6.20            | 8.50             | 9.55         |
| Insoluble matter in the first trichloroacetic acid precipitate | 8.52 | 1.43 | 0.05 | 0.19 | 1.28           | 0.82             | 0.00         |
| PPD (trichloroacetic acid precipitate) | 22.12 | 9.60 | 3.90 | 7.60 | 1.38           | 5.36             | 1.19         |
| Trichloroacetic acid supernatant | 20.26 | 25.70 | 31.75 | 42.32 | 35.70           | 17.24            | 27.11        |
| Washings*                     | 2.77            | 2.08         | 1.36     | 3.09        | 2.70            | 2.71             | 1.79         |

*a* Recovery of radioactivity, expressed in percentage of the radioactivity added to the culture medium.  
*b* Washings of cells and of PPD (trichloroacetic acid precipitate).

### Table 4. Specific radioactivity of ¹⁴C-labeled substances obtained during the growth of various mycobacteria

| Determination                  | M. tuberculosis | M. bovis BCG | M. avium | M. kansasii | M. scrofulaceum | M. intracellulare | M. fortuitum |
|-------------------------------|-----------------|--------------|----------|-------------|-----------------|------------------|--------------|
| Cells                         | 56,000¹         | 65,240       | 107,000  | 30,800      | 168,500         | 53,700           | 47,680       |
| Carbon dioxide                | 8,650           | 7,080        | 7,800    | 6,900       | 5,160           | 6,940            | 2,910        |
| PPD (trichloroacetic acid precipitate) | 168,500 | 145,800 | 137,900 | 120,500 | 329,000         | 177,000          | 124,100      |
| PPD (ammonium sulfate precipitate) | 173,060 | 145,050 | 145,050 | 180,770 |               |                  |              |
| Protein*                      | 174,000         | 147,500      | 174,800  | 146,500     | 342,000         | 182,000          | 127,100      |
| Nucleic acid*                 | 48,900          | 37,400       | 67,200   | 24,300      | 37,750          | 51,300           | 33,300       |
| Insoluble matter in the first trichloroacetic acid precipitate | 92,600 | 150,000 | 39,800 | 38,200 | 342,000         | 84,200           |              |

*a* All specific activities are expressed in counts per minute per milligram of substance. With a counter-efficiency of 30.8%, 168,500 counts per min per mg (= 547,078 dpm/mg) equals 0.25 μCi/mg.  
*b* Obtained after paper-electrophoretic separation from PPD (trichloroacetic acid precipitate).
radioactivity by increasing the original radioactivity or by decreasing the volume of the culture medium.

**Distribution of radioactivity between protein and nucleic acid in ^14^C-PPD produced from various species of mycobacteria.** The main impurity of tuberculin PPD prepared by trichloroacetic acid precipitation is nucleic acid and its amount is known to vary greatly between different lots of PPD (3). Since the nucleic acid contained in the preparations of ^14^C-tuberculin PPD was also radioactive, it was of interest to determine the distribution of radioactivity between protein and nucleic acid in the preparations of ^14^C-PPD from various species of mycobacteria. Table 5 shows that, in PPD-BCG, PPD-A, PPD-G, and PPD-F obtained by trichloroacetic acid precipitation, the radioactivity due to nucleic acid content was well below 1% of the total radioactivity of the preparations. Therefore, no further purification was done.

**Table 5. Distribution of weight and radioactivity between protein and nucleic acid in ^14^C-PPD**

| Type of antigen | Fraction         | Specific radioactivity^a^ | PPD (trichloroacetic acid precipitate) | PPD (ammonium sulfate precipitate) |
|----------------|------------------|---------------------------|----------------------------------------|------------------------------------|
|                |                  |                           | Weight^b^ | Radioactivity^b^ | Weight^b^ | Radioactivity^b^ |
| Tuberculin PPD | Protein          | 174,000                   | 95.62     | 98.73         | 99.25     | 99.79           |
|                | Nucleic acid     | 48,900                    | 4.38      | 1.27          | 0.75      | 0.21            |
| PPD-BCG        | Protein          | 147,500                   | 98.44     | 99.61         |           |                |
|                | Nucleic acid     | 37,400                    | 1.56      | 0.39          |           |                |
| PPD-A          | Protein          | 174,800                   | 99.18     | 99.69         |           |                |
|                | Nucleic acid     | 67,200                    | 0.82      | 0.31          |           |                |
| PPD-Y          | Protein          | 146,500                   | 78.50     | 95.66         | 98.81     | 99.80           |
|                | Nucleic acid     | 24,300                    | 21.50     | 4.34          | 1.19      | 0.20            |
| PPD-G          | Protein          | 342,000                   | 95.81     | 99.52         |           |                |
|                | Nucleic acid     | 37,750                    | 4.19      | 0.48          |           |                |
| PPD-B          | Protein          | 182,000                   | 96.44     | 98.97         | 99.06     | 99.73           |
|                | Nucleic acid     | 51,300                    | 3.56      | 1.03          | 0.94      | 0.27            |
| PPD-F          | Protein          | 127,100                   | 96.75     | 99.13         |           |                |
|                | Nucleic acid     | 33,300                    | 3.25      | 0.87          |           |                |

^a^ Expressed as counts per minute per milligram.
^b^ Percentage of PPD.

**Table 6. Specific radioactivity and relative potency of seven ^14^C-PPD antigens used to study the adsorption of these antigens to glass**

| Type of antigen | Prepn            | Specific radioactivity^a^ | Relative potency (nonradioactive PPD taken as 1.00) |
|----------------|-----------------|---------------------------|-----------------------------------------------|
|                |                 | Counts per min per mg     | Micro-curies/ mg                               |
| ^14^C-tuberculin PPD | Ammonium sulfate precipitate | 173,060 | 0.25 | 0.879 (0.618–1.231)^c |
| ^14^C-PPD-BCG   | Trichloroacetic acid precipitate | 145,800 | 0.21 | 0.716 (0.536–0.932) |
| ^14^C-PPD-A     | Trichloroacetic acid precipitate | 173,900 | 0.25 | 2.611 (1.590–5.501)^d |
| ^14^C-PPD-Y     | Ammonium sulfate precipitate | 145,050 | 0.21 | 1.484 (1.142–1.985) |
| ^14^C-PPD-G     | Trichloroacetic acid precipitate | 329,000 | 0.48 | 0.848 (0.561–1.247) |
| ^14^C-PPD-B     | Ammonium sulfate precipitate | 180,770 | 0.26 | 1.333 (1.020–1.782) |
| ^14^C-PPD-F     | Trichloroacetic acid precipitate | 124,100 | 0.18 | 0.677 (0.541–0.832) |

^a^ Counting efficiency of 30.8% was used to convert counts per minute into microcuries, and the average specific radioactivity was 0.26 µCi per mg of ^14^C-PPD.
^b^ Relative potencies were determined by comparison with nonradioactive PPD of the same species of mycobacteria (tuberculin PPD, PPD-BCG, PPD-A, PPD-Y, PPD-G, PPD-B, and PPD-F) taken as 1.00 and are the results of statistical evaluation. The figures in brackets represent 95% confidence limits.
^c^ Relative potency was determined by comparison with the United States reference standard tuberculin PPD (PPD-S; 1).
^d^ Relative potency was determined by comparison with the international standard PPD for avian tuberculin (Central Laboratories, New Haw, Weybridge, Surrey, England).
However, in PPD-Y, PPD-B, and tuberculin PPD, the radioactivity due to nucleic acid was above 1%, and therefore these preparations were further purified by redissolving them in 0.07 M NaHPO₄ solution and reprecipitating them with an equal volume of saturated ammonium sulfate (pH 7.0) to remove most of the nucleic acid. The radioactivity due to nucleic acid in these PPD preparations was now also well below 1% of the total radioactivity of the preparations (Table 5).

**Biological activity of **¹⁴C-PPD **from various species of mycobacteria.** Skin tests on seven groups of guinea pigs sensitized with the corresponding species of heat-killed mycobacteria showed that all of the seven ¹⁴C-labeled PPD preparations were biologically active. Their potency relative to the potency of nonradioactive PPD of the same type was of the same order (Table 6).

**Adsorption to glass of **¹⁴C-labeled PPD obtained from various mycobacteria: unstabilized PPD solutions.** Previous studies have shown that ¹⁴C-tuberculin PPD can be used to measure the adsorption of tuberculoprotein to glass and plastic surfaces (4). The specific radioactivity of the various ¹⁴C-PPD antigens used in the following adsorption studies was on the average 0.26 µCi/mg of dry powder ¹⁴C-PPD (0.18 to 0.48 µCi/mg; Table 6). Glass ampoules were preferred since they had been found to give more consistent results than glass vials closed with a rubber stopper. The method employed was as follows. Sets of 30 10-ml glass ampoules were filled with 2.5 ml of ¹⁴C-PPD solution (0.001 mg/ml of PPD). After various times of storage at 5°C, two ampoules were withdrawn and tested for their radioactivity (¹⁴C-PPD) in solution.

The results of adsorption studies over a period of 12 months are given in Table 7. These data show, first, that there is no significant difference in the adsorption properties of the tuberculin PPD obtained from *M. tuberculosis* and the PPD obtained from six other species of mycobacteria (*M. bovis* BCG, *M. avium, M. kansasi, M. scrofulaceum, *M. intracellulare*, and *M. fortuitum*); second, regardless of the origin of the PPD, a loss of about 40% in radioactivity took place during the first 8 hr, whereas after 1 week a loss of approximately 70 to 80% had occurred. At 1 month the loss was of the order of 90% and thereafter remained practically constant for the duration of the storage period (Table 7).

**PPD solutions stabilized with Tween 80.** Previous studies have shown (8) that adsorption of tuberculin PPD to glass could be prevented by

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**Table 7. Adsorption of ¹⁴C-PPD from various mycobacteria to glass ampoules a without and with addition of 0.0005% Tween 80**

| Storage Time | M. tuberculosis (tuberculin PPD) | M. bovis BCG (PPD-BCG) | M. avium (PPD-A) | M. kansasi (PPD-Y) | M. scrofulaceum (PPD-G) | M. intracellulare (PPD-B) | M. fortuitum (PPD-F) |
|--------------|---------------------------------|------------------------|------------------|-------------------|------------------------|--------------------------|-----------------------|
|              | Control (0.0005%)                | Control (0.0005%)       | Control (0.0005%)| Control (0.0005%) | Control (0.0005%)       | Control (0.0005%)         | Control (0.0005%)       |
| 8 hr         | 63 93 78                         | 61 93 88              | 63 93 78         | 71 96 76           | 76 89 58               | 58 100                   |
| 1 day        | 60 97 80                         | 65 94 88              | 62 95 60         | 60 98 59           | 59 98 98              | 44 71                    |
| 1 week       | 29 89 74                         | 30 88 77              | 20 97 75         | 25 98 19           | 19 97 77              | 15 81                    |
| 2 weeks      | 22 88 72                         | 14 88 72              | 15 97 85         | 21 88 15           | 15 93 71              | 8 88                     |
| 1 month      | 13 84 10                         | 14 89 90              | 9 89 13          | 11 93 14           | 13 91 11              | 13 93                    |
| 3 months     | 12 84 6                          | 14 89 90              | 7 86 10          | 14 89 14           | 13 91 11              | 13 93                    |
| 6 months     | 9 87 5                           | 15 85 8              | 8 95 9           | 10 88 14           | 14 89 14              | 13 93                    |
| 9 months     | 7 79 5                           | 12 79 5               | 8 78 7           | 8 88 7             | 7 84 8                | 7 84                     |
| 12 months    | 9 84 4                           | 16 81 4               | 6 86 6           | 11 87 9            | 11 87 9               | 11 87                    |
| Avg 1 month  | 10.0 83.6                        | 6.0 67.6              | 6.2 91.8         | 16.0 84.0          | 6.6 90.0              | 9.2 85.2                 |
| to 12 months |                                |                       |                  |                   |                       |                          |

a Ampoules contained 2.5 ml of ¹⁴C-PPD solution (0.001 mg of PPD per ml; 0.3% phenol; phosphate-buffered saline, pH 7.38) and were stored at 5°C.
the addition of an antiadsorption agent. Certain surface-active and colloidal substances possess this beneficial antiadsorption property; for instance, Tween 80 at the concentration of 0.0005% stabilized tuberculin PPD obtained from *M. tuberculosis* by preventing to a great extent the adsorption of tuberculoprotein to glass (4). It was therefore assumed that Tween 80 at the same concentration would also stabilize PPD produced from mycobacteria other than *M. tuberculosis*. To verify this assumption, the same 14C-PPD antigens used in the adsorption studies above described were stabilized by the addition of 0.0005% Tween 80 to the PPD solution, and the radioactivity in solution was determined over a period of 12 months.

Table 7 shows that Tween 80 (0.0005%) prevented the adsorption to glass of PPD-A, PPD-Y, PPD-G, PPD-B, and PPD-F to the same degree as it prevented the adsorption of tuberculin PPD. However, it is worthwhile to point out that the PPD-BCG, obtained from *M. bovis* BCG, for some unexplained reasons was not so effectively prevented from being adsorbed to glass even when the Tween 80 concentration was increased by 10-fold to 0.005% (Table 8).

**Conclusion.** 14C-PPD antigens can be obtained by incorporating a pool of 14C-amino acids in the culture medium. All of these PPD antigens derived from different species of mycobacteria adsorb equally well to glass surfaces. The addition of 0.0005% Tween 80 to dilute solutions of these PPD antigens prevented most of the PPD from being adsorbed to the wall of the glass container. It can therefore be concluded that the addition of Tween 80 at the concentration of 0.0005% could also be recommended for PPD antigens produced from mycobacteria other than *M. tuberculosis*.

**ACKNOWLEDGMENTS**

This investigation was supported by a grant from the National Sanitarium Association, Muskoka Hospital Memorial Research Fund Committee, Toronto, Ontario, Canada. We are grateful to S. Ober and L. Jais for excellent technical assistance.

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