Preparation, Purification, and Stability of Tuberculin

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ABSTRACT

LANDI, S. (University of Toronto, Toronto, Canada). Preparation, purification, and stability of tuberculin. Appl. Microbiol. **11**:408–412. 1963.—The method used to produce "Connaught" tuberculin purified protein derivative (PPD) is described. The tuberculin PPD for the multiple-puncture method was shown to be stable for at least 24 months at 5 C; tuberculin PPD for the intracutaneous method was shown to be stable at 5 C and 24 C for a period of 18 months in the presence of Tween 80. Evans blue or brillant vital red was added to tuberculin PPD for improved testing by the multiple-puncture method. These tinted tuberculin preparations were found to be as stable as the Connaught tuberculin PPD preparations without dye at 5 C. Freeze-dried tuberculin PPD with Plasdone as an inert base was found to be remarkably stable for a period of at least 24 months at 5, 24, and 37 C.

The ever-increasing demand for a purified form of tuberculin has stimulated interest in its preparation and in the investigation of its properties. The importance of the tuberculin skin test in tuberculosis prevention programs underlines the need for the preparation of a purified tuberculin with adequate stability. The present work deals with the purification of tuberculin and its stability for preparations used for the multiple-puncture (Heaf, 1951) and the intracutaneous (Mantoux, 1909; National Tuberculosis Association, 1961) methods. For this purpose, the purified tuberculin was tested after storage at various temperatures.

It was thought that the addition of a dye to the tuberculin preparation used for the multipuncture method would facilitate the tuberculin test and increase its accuracy. It was necessary, therefore, to determine the effect of the dye on the stability of the preparation as compared with the stability of an identical preparation without dye.

It was also thought of interest to determine the effect of freeze-drying on the potency and stability of purified tuberculin.

MATERIALS AND METHODS

Organism and medium. The "Johnston" strain of *Mycobacterium tuberculosis* var. *hominis*, isolated in 1920 in Toronto, Canada, from a case of human tuberculosis, was used for the production of tuberculin purified protein derivative (PPD). The "Johnston" strain showed the characteristic surface growth of *M. tuberculosis* var. *hominis* on Long's synthetic medium (Long and Seibert, 1926) as well as virulence for guinea pigs and mice and nonvirulence for rabbits. The cultures of the organism were maintained by weekly subculture on Long's synthetic medium.

For tuberculin production, the medium was dispensed in 150-ml amounts into Roux bottles. These bottles were sterilized in an autoclave by treatment for 10 min in flowing steam, followed by 30 min at a steam pressure of 15 lb/in.^{2}

Preparation of tuberculin. The organisms for inoculation were grown as a surface pellicle in small Erlenmeyer flasks on Long's synthetic medium at 37 ± 0.5 C for 8 to 10 days. The pellicle was used for inoculating approximately 300 Roux bottles of medium. After inoculation, these bottles were incubated at 37 ± 0.5 C for 6 to 6.5 weeks. When the culture was ready to be harvested, it was tested for purity by transferring 0.50 ml from each Roux bottle into 10 ml of beef infusion broth and incubating at 37 ± 0.5 C. If contamination appeared in any tube, the contents of the corresponding bottle were discarded.

After the Roux bottles had been tested for purity, they were steamed for 3 hr in a flowing-steam cabinet (100 C) and allowed to stand overnight at room temperature. The contents of the bottles were then passed through a sterile eight-layer gauze pad or a flannelette cloth placed over a filter-paper pulp pad to separate the medium containing tuberculoprotein from the bacterial growth. The crude filtrate, light amber in color, was then filtered through a Berkefeld candle. A sterility test was carried out on this filtrate, and phenol was added to it as a preservative (0.35%, final concentration). The filtrate was then stored at refrigerator temperature until purification time.

Purification of tuberculin. Following the method of Green (1946), 40% trichloroacetic acid was added to the filtrate to give a final concentration of 4%. The total protein was precipitated and allowed to sediment overnight in a refrigerator (5 C); the supernatant was siphoned off and discarded. The sedimented sludge was poured into 250-ml centrifuge cups and centrifuged at 840 × *g* for 20 min. The sludge was washed with 1% trichloroacetic acid and centrifuged again at 1880 × *g* for 15 min. The residue was washed again with 1% trichloroacetic acid and centrifuged at 3350 × *g* for 15 min, and finally washed with cold 0.30% KH_2PO_4 and centrifuged at 4000 rev/min for

30 min. The washed precipitate was dissolved in 0.47% Na_2HPO_4 containing 0.50% NaCl to give a volume equal to approximately 0.04 of the original volume of the filtrate. This slightly turbid solution was clarified by centrifugation at 16,000 rev/min in a Servall type RC-Z refrigerated centrifuge (4 C) for 4 hr. The supernatant was decanted and passed through a Berkefeld candle. Sterility tests were then carried out, as well as an infectivity test on three normal guinea pigs, to ascertain the absence of living tubercle bacilli. Phenol was added to the tuberculoprotein solution as a preservative (0.50% phenol, final concentration). This formed a tuberculin PPD, designated "Connaught" tuberculin PPD stock solution, from which appropriate dilutions could be made.

Standardization of tuberculin PPD. The stock solution of tuberculin PPD was tested for nitrogen content by the micro-Kjeldahl method, and the amount of protein was calculated [mg of nitrogen × factor (6.25) = mg of protein per sample].

Preparations from the stock solution of "Connaught" tuberculin PPD were assayed against the NIH Reference Standard Tuberculin PPD (obtained each year from the National Institutes of Health, Bethesda, Md., as a solution of 1 mg of active PPD in buffered saline containing 0.50% phenol as preservative) by a method based on the size of the skin reaction produced when preparations were injected intradermally into Bacillus Calmette-Guérin (BCG)-sensitized guinea pigs. Four or eight guinea pigs were used for the assay. The animals were sensitized by two intradermal injections of 0.1 mg of fresh or freeze-dried BCG (prepared by the author in the Connaught Medical Research Laboratories) at least 1 month prior to the use in the potency test. The animals were closely clipped over the back and sides and then depilated with barium sulfide before the intradermal tests were made.

The flanks of each guinea pig were divided into four squares. Two dose levels of standard and unknown were assigned in duplicate to the eight positions on each animal in a random fashion. The dose injected intradermally was 0.1 ml at each level of standard and unknown [20 tuberculin units (TU) and 5 TU unless otherwise specified]. At 24 hr after the injections, the diameter of each reaction zone was measured, and the result was recorded as the sum of two perpendicular diameters in millimeters.

The estimate of potency was made by the method of Long, Miles, and Perry (1954). The tuberculin was of acceptable potency if statistical analysis of the test results showed the tuberculin PPD content to lie within 80 to 120% of the standard.

Results and Discussion

Stability of tuberculin PPD for the multiple-puncture method. The "Connaught" tuberculin PPD used for the stability tests was standardized as described for the stock solution and was found to be 86% as potent as the NIH standard. It was filled in 1-ml hard-glass vials, with tightly screwed caps, and sealed and stored at refrigerator temperature (5 C), at room temperature, and at 37 C. The material tested contained the following ingredients: an amount of tuberculin PPD biologically equivalent to 2 mg/ml of the NIH Reference Standard Tuberculin PPD, 50% glycerine (by weight), 0.24% Na_2HPO_4, 0.50% NaCl, and 0.50% phenol; pH 7 to 7.4.

Table 1 shows that there was no significant trend in the relative potencies at refrigerator temperature (5 C) for a period of 24 months. These relative potencies did not differ significantly from the initial relative potency (0.86). However, there was a significant trend in the relative potencies at room temperature and at 37 C, indicating a slight loss of potency.

Effect of added dyes on the stability of tuberculin PPD for the multiple-puncture method. It was felt that the addition of a dye to the tuberculin PPD used for the multiple-puncture method would help the operator to locate more readily the area where the tuberculin had been applied, thus facilitating the test and increasing its accuracy. The effect of dyes such as Evans blue and brilliant vital red on the stability of "Connaught" tuberculin PPD was, therefore, investigated.

Preparations of tuberculin with dye were compared with the very same batches of tuberculin without dye, with the latter tuberculin as standard. These preparations, as well

TABLE 1. *Stability of "Connaught" tuberculin PPD for the multiple-puncture method**

Storage temp	Storage time (months)					
	6	7	12	15	18	24
5 C	0.94 (0.71–1.26)	0.83 (0.67–1.04)	0.82 (0.63–1.07)	1.06 (0.70–1.60)	0.88 (0.65–1.19)	1.12 (0.79–1.59)
Room temp	1.19 (0.88–1.59)	0.77 (0.62–0.97)	0.75 (0.57–0.97)	0.98 (0.65–1.47)	0.68 (0.50–0.93)	0.70 (0.48–1.01)
37 C	0.93 (0.70–1.25)	0.78 (0.62–0.98)	0.56 (0.43–0.74)	0.82 (0.54–1.24)	0.60 (0.44–0.81)	0.69 (0.47–1.00)

* Results are expressed as potencies relative to NIH Standard. Numbers in parentheses are 95% limits. Initial relative potency was 0.86 (0.75–0.99).

TABLE 2. *Effect of added Evans blue on the stability of "Connaught" tuberculin PPD for the multiple-puncture method**

Storage temp	Storage time (months)					
	3	6	9	12	15	18
C						
5	1.05 (0.69–1.61)	1.22 (0.70–2.13)	0.88 (0.73–1.06)	1.16 (0.91–1.50)	1.06 (0.71–1.59)	0.84 (0.61–1.17)
24†	1.08 (0.91–1.29)	0.92 (0.71–1.18)	1.51 (1.01–2.30)	0.81 (0.58–1.12)		
37†	0.84 (0.68–1.04)	0.85 (0.66–1.09)	0.76 (0.50–1.13)	1.06 (0.77–1.46)		

* Results are expressed as potencies relative to "Connaught" tuberculin PPD (maintained under the same conditions as "Connaught" tuberculin PPD with Evans blue) relative potency. Numbers in parentheses are 95% limits. Initial relative potency was 0.94 (0.81–1.08).

† Prior to testing, this tuberculin was kept at 5 C for 6 months.

TABLE 3. *Effect of added brilliant vital red on the stability of "Connaught" tuberculin PPD for the multiple-puncture method**

Storage temp	Storage time (months)			
	3	6	9	12
C				
5	1.19 (0.89–1.58)	1.25 (0.91–1.71)	0.86 (0.62–1.20)	1.28 (0.93–1.77)
24	1.26 (0.94–1.67)	0.94 (0.69–1.30)	1.07 (0.77–1.49)	0.93 (0.67–1.27)
37	1.11 (0.84–1.48)	1.11 (0.81–1.52)	0.94 (0.67–1.30)	1.18 (0.86–1.63)

* Results are expressed as potencies relative to "Connaught" tuberculin PPD (maintained under the same conditions as "Connaught" tuberculin PPD with brilliant vital red) relative potency. Numbers in parentheses are 95% limits. Initial relative potency was 0.83 (0.60–1.14).

as the tuberculin used as standard, were filled in 5-ml Pyrex test tubes, tightly stoppered, sealed, and stored at refrigerator temperature (5 C), at 24 C, and at 37 C. The material tested contained the following ingredients. Tuberculin PPD with Evans blue contained an amount of tuberculin PPD biologically equivalent to 2 mg/ml of the NIH Reference Standard Tuberculin PPD, 50% glycerine (by weight), 0.91% KH_2PO_4, 0.95% Na_2HPO_4, 0.50% NaCl, and 20 mg/100 ml of Evans blue (British Drug Houses Ltd., Poole, England); pH 7.20. Tuberculin PPD with brilliant vital red contained the same ingredients as above, but Evans blue was replaced with 40 mg/100 ml of brilliant vital red (Allied Chemical Corp., National Aniline Division).

The effects of the dyes on the stability of the two tinted preparations are reported in Tables 2 and 3. These results show that the stability of tuberculin PPD with Evans blue was not altered for a period of at least 18 months at 5 C and for a period of at least 12 months at 24 and 37 C. These results also show that the stability of tuberculin PPD with brilliant vital red was not altered for a period of at least 12 months at 5, 24, and 37 C. These data reveal, therefore, that the dyes used had no deleterious effect on the potency of tuberculin PPD, and that the tinted products maintained their stability as well as the tuberculin PPD without dye. Therefore, it appears that the use of a tinted tuberculin PPD would improve the multiple-puncture method of skin testing. These materials have been used in human beings and no tattooing effect has been found.

Stability of tuberculin PPD for the intracutaneous method. Various strengths of tuberculin PPD for the intracutaneous method were prepared from a stock solution of "Connaught" tuberculin PPD to give 10, 50, and 1000 TU per ml (0.0001, 0.0005, and 0.01 mg of PPD, respectively). The diluent used to make these preparations was prepared according to Magnusson et al. (1958) and contained Tween 80 as a stabilizing agent. These various strengths of tuberculin PPD were standardized against the NIH Reference Tuberculin PPD Standard, which was diluted, just prior to testing, with the same diluent used to prepare the three strengths of "Connaught" tuberculin PPD. The solutions of Connaught tuberculin PPD were placed at refrigerator temperature (5 C) and at 24 C. Stability tests were carried out from time to time over a period of 18 months. The Reference Standard was kept at refrigerator temperature at a strength of 50,000 TU per ml.

The material tested contained the following ingredients: an amount of tuberculin PPD biologically equivalent to 10, 50, and 1000 TU per ml of NIH Reference Standard Tuberculin PPD, 1.45 g of KH_2PO_4, 7.60 g of Na_2HPO_4 (Magnusson used $Na_2HPO_4,2H_2O$), 4.80 g of NaCl, 0.01% Chinosol as a preservative (M-299, Benzol Products Co., Newark, N.J.), 0.005% Tween 80 as the stabilizing agent, and distilled water to a total volume of 1050 ml.

Table 4 shows that there was no significant trend in the relative potencies at refrigerator temperature (5 C) and

TABLE 4. *Stability of "Connaught" tuberculin PPD for the intracutaneous method**

PPD dilution	Initial potency	Storage temp	Storage time (months)			
			6	9	15	18
TU/ml		*C*				
1000	1.28 (0.98–1.66)	5	1.02 (0.84–1.23)	1.48 (0.81–2.71)	1.36 (0.94–1.96)	1.20 (0.72–2.06)
		24	0.82 (0.68–1.00)	1.06 (0.59–1.91)	1.21 (0.84–1.75)	1.29 (0.77–2.23)
50	1.15 (0.85–1.54)	5	0.95 (0.59–1.52)	0.74 (0.43–1.28)	1.21 (0.68–2.16)	1.17 (0.55–2.66)
		24	0.71 (0.44–1.15)	0.89 (0.51–1.52)	0.71 (0.40–1.28)	1.78 (0.85–4.46)
10	1.02 (0.82–1.20)	5	1.11 (0.86–1.44)	0.77 (0.39–1.52)	1.39 (0.95–2.03)	0.87 (0.75–1.01)
		24	0.80 (0.61–1.03)	0.73 (0.37–1.45)	1.36 (0.93–1.99)	0.90 (0.77–1.04)

* Results are expressed as potencies relative to NIH Standard. Numbers in parentheses are 95% limits.

TABLE 5. *Stability of "Connaught" tuberculin PPD, freeze-dried, with Plasdone as an inert base for the intracutaneous method**

Storage temp	Storage time (months)				
	3	6	12	18	24
C					
5	0.97 (0.63–1.48)	0.94 (0.58–1.54)	1.16 (0.60–2.23)	1.00 (0.59–1.71)	0.93 (0.66–1.30)
24	0.78 (0.51–1.20)	0.78 (0.48–1.28)	1.31 (0.68–2.53)		1.10 (0.62–1.96)
37	0.93 (0.61–1.43)	1.17 (0.72–1.90)	2.00 (1.03–3.87)		1.00 (0.70–1.43)

* Results are expressed as potencies relative to NIH Standard. Numbers in parentheses are 95% limits. Initial relative potency was 1.18 (0.67–2.06).

at 24 C for a period of 18 months. These relative potencies did not differ significantly from the initial relative potencies.

Stability of tuberculin PPD, freeze-dried, for the intracutaneous method. Freeze-dried tuberculin PPD is believed to be stable for at least several years. It was, therefore, the purpose of this experiment to investigate the effect of freeze-drying on the potency of "Connaught" tuberculin PPD to determine the stability of the freeze-dried preparation at different temperatures (5, 24, and 37 C). The data reported in Table 5 were collected over a period of 24 months, although this experiment was intended for a much longer period of time; and further data on the stability of this material will be reported at a later date.

The material tested contained the following ingredients: an amount of tuberculin PPD biologically equivalent to 1000 TU per ml and 50 TU per ml of the NIH Reference Standard Tuberculin PPD, 1% Plasdone (pharmaceutical polyvinylpyrrolidone; K 26-28, Chemical Developments of Canada) used as an inert base to incorporate the tuberculin, 0.85% NaCl, 0.25% Na_2HPO_4, and 0.057% NaH_2PO_4; pH 7.29.

Amounts of 2 ml of each strength of tuberculin PPD were dispensed separately into 10-ml vials. These vials were partially closed by a grooved rubber stopper and were placed in a deep freeze (− 30 C) for approximately 3 hr. They were then transferred to a freeze-drying unit and maintained there for about 20 hr.

The freeze-dried tuberculin has a very white fluffy appearance. After storage for 24 months at 5, 24, and 37 C this material showed no change in appearance.

Stability tests on the freeze-dried material were carried out at tuberculin concentrations of 1000 and 50 TU/ml. These strengths were compared with the NIH Reference Standard Tuberculin PPD, freshly diluted in buffered saline, but containing no Plasdone, as it was found that the presence of Plasdone in the NIH Reference Standard did not alter its potency as compared with the tuberculin PPD standard without Plasdone.

Table 5 shows that freeze-dried tuberculin PPD was remarkably stable at 5, 24, and 37 C for a period of 24 months.

In these studies the method used by Green (1946), as modified in the Connaught Medical Research Laboratories, was shown to be suitable for the production of tuberculin PPD. Long's synthetic medium (Long and Seibert, 1926) replaced the medium employed by Green (1946), and only one strain of *M. tuberculosis*, the "Johnston" strain, was used instead of the three strains, C., D.T., and P.N.

"Connaught" tuberculin PPD for the multiple-puncture method was shown to be very stable. "Connaught" tuber-

culin PPD for the Mantoux test, containing Tween 80 as a stabilizing agent, was also shown to be very stable.

The addition of a dye such as Evans blue or brilliant vital red, for improved testing by the multiple-puncture method, had no deleterious effect on the tuberculin PPD potency, and the preparation showed the same degree of stability as the tuberculin PPD without dye. No tattooing effect was encountered on human beings skin-tested with tinted tuberculin PPD.

The "Connaught" freeze-dried tuberculin PPD is remarkably stable, and the use of Plasdone in the freeze-dried preparation has proved of value as an inert base.

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