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PREPARATION OF PURIFIED TUBERCULIN RT 23

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SYNOPSIS

The technical procedure used in the preparation of a batch of more than 500 g of purified tuberculin (PPD) is described. This batch is designated RT 23, and it is estimated that the quantity now prepared will cover the global demand for purified tuberculin for human use for several years.

RT 23 has been prepared by mixing 77 smaller lots of tuberculin selected from a total of 95 lots. The method of preparing the individual lots is described and the experimental data, i.e., the yield and the biological activity ascertained by skin tests in BCG-vaccinated guinea-pigs, are given for all lots.

The possible causes of variations in the yield and biological activity of the individual lots are discussed.

Various preparations of tuberculin, whether old tuberculin or purified tuberculin, are known to vary in potency, and it is reasonable to assume that other important properties, such as antigenicity, specificity and stability, may vary also. Furthermore, it is very difficult to measure and control such variations in different preparations of tuberculin, so that there is no doubt that varying or incomparable results of tuberculin testing are obtained when different preparations are used. Thus, it would be an advantage to have one large batch of tuberculin available for general use over a longer period.

Accordingly, it was agreed in 1955 between UNICEF and the Statens Seruminstitut, Copenhagen, that a large batch of purified tuberculin (PPD) should be prepared at the Statens Seruminstitut and that UNICEF should make this preparation available for general use for intradermal tuberculin tests in humans. The amount prepared was to be at least 500 g —an amount which it was estimated would cover the global demand for purified tuberculin for 10 years.

It was further agreed that, as far as technically possible, the tuberculin should be prepared by the same method as previous batches of purified tuberculin from the Statens Seruminstitut (RT 19-21, RT 22), such tuberculin having proved to be suitable for large-scale use.

The purpose of the present paper is to describe the technical procedure used in the preparation of this batch, and to report the experimental results obtained during the process.

The preparation is designated " Purified Tuberculin RT 23 " and has been prepared by mixing many small lots of tuberculin, each prepared separately. A description is given of the method of preparing and mixing the individual lots. The biological standardization of RT 23 is described in a separate article.[1] The preparation has been issued since 1 July 1958.

Preparation of Individual Lots

Technical procedure

Medium. Sauton medium, as modified slightly by Lind (1947), was used. The composition of the medium is as follows:

Asparagine	4 g
Citric acid	2 g
Potassium monohydrogen phosphate	0.5 g
Magnesium sulfate	0.5 g
Ferric ammonium citrate	0.05 g
Glycerol	60 ml
Tap water	950 ml
Concentrated ammonia for adjustment of the pH to	6.8-6.9

The medium was distributed in volumes of 1 litre in 1.5-litre flasks and sterilized by autoclaving at 120°C for 20 minutes. The pH of the medium was 7.2-7.4 when measured after sterilization.

Strains. Seven different virulent strains of *Mycobacterium tuberculosis*, human type, were used.

Four of the strains — E 5, E 9656, E 1580 and U 1921—were selected as those which grew best out of six old laboratory strains isolated more than ten years ago. These strains had been used previously for the preparation of purified tuberculin at the Statens Seruminstitut (RT 19-21 and RT 22).[2]

The three other strains—T 3419, T 3480 and T 3487—were isolated from tuberculosis patients shortly before being used for production of tuberculin.

The virulence of all the strains was tested before production was initiated, and again on conclusion of the process, by subcutaneous injection of 10^{-5} mg of culture into guinea-pigs. All the animals developed generalized tuberculosis within eight weeks.

[1] See page 845 of this number of the *Bulletin*.

[2] Studies on the production of tuberculin with three of these strains have previously been published by Lind (1948).

The strains were subcultured as surface cultures on the modified Sauton medium every fortnight.

Inoculation, incubation and sterilization of bacterial cultures. The surface of the medium in each culture flask was inoculated with a bacillary pellicle about 10 mm in diameter. The cultures were incubated at 38°C for 4½ to 5½ weeks. After incubation the cultures were sterilized by heating in streaming steam for one hour.

Filtration of bacterial cultures. Usually, 150 to 200 one-litre flasks were filtered at a time. Most of the killed tubercle bacilli were filtered off through a double layer of gauze. The remaining tubercle bacilli were removed by Seitz filtration (filter plates K_0) followed by Berkefeld filtration. The sterile culture filtrates were stored in 50-litre aluminium containers.

Concentration and precipitation of tuberculin. The culture filtrates were concentrated by ultrafiltration against collodium filters. The filtration plant consisted of eight porcelain kidney filters in an open stainless-steel container. The kidney filters were covered by collodium membranes, prepared from a 7.5% solution of Parlodion [1] in glacial acetic acid.

The ultrafiltration was carried out at 0-2°C, the culture filtrates being covered by a layer of toluene during the process, in order to minimize the risk of contamination.

Generally, 100 litres of culture filtrate were concentrated for one lot of PPD. The rate of ultrafiltration was 50 litres per day, and after the concentration the culture filtrate was washed with 50 litres of sterile distilled water. The final volume of the concentrate was 0.2-4 litres.

The toluene was removed from the concentrated culture filtrate by filtration through moistened paper. Finally, the tuberculin was precipitated by addition of trichloracetic acid to a final concentration of 2%. After sedimentation for half an hour, the precipitate was centrifuged off and washed four times with 2% trichloracetic acid.

Dehydration of purified tuberculin. Dehydration of the PPD was effected by stirring the precipitate repeatedly with anhydrous ether. Usually, about 4-6 litres of ether were used for desiccation of a lot of 5-10 g of PPD.

After dehydration the yield was measured by weighing. The individual lots were stored at room temperature until used for mixing.

Control of sterility

Two requirements as regards sterility are essential in connexion with the preparation of tuberculin. First, the tuberculin dilutions must be sterile when used for injecting, and consequently the PPD powder must

[1] Mallinckrodt Chemical Works, New York, USA.

be sterile. Secondly, the tuberculin should as far as possible be kept sterile during the production process, or at any rate the growth of bacteria must be prevented. If precautions in this direction are not taken, the tuberculin may contain foreign antigens, which may increase the pyrogenicity of the product or give rise to non-specific reactions in population groups sensitized against them.

The sterility tests carried out on the finished product showed the latter to be sterile, despite the fact that, as mentioned later, contamination was occasionally demonstrated during the preparation of the individual lots. Apparently, the precipitation with trichloracetic acid, combined with the dehydration with ether and the treatment with ether during the mixing process, has caused the final product to be sterile.

As regards sterility during the production process, mention will be made first of the sterility control carried out during the culture of the tubercle bacilli, and then of the results of the sterility tests performed during the production process itself (filtration and concentration).

All the mother cultures used for inoculation were found to be pure cultures. During incubation the bacterial cultures were carefully observed, and contaminated (turbid or discoloured) cultures discarded. It is estimated that an average of 1-2% of the cultures were contaminated. For the first lots, the reliability of the macroscopic observation was checked by direct sterility tests on about 200 cultures, but no further contamination was found.

Sterility tests of the individual lots at different stages of the production process, particularly during ultrafiltration, showed that there was contamination in some cases. This was caused by a number of different bacteria, especially Gram-positive rods, motile or non-motile Gram-negative rods, and Gram-positive diplococci. Some of these contaminants could grow only at room temperature (25°C), incubation at 37°C giving no growth. The contamination was often found only after incubation for 4-10 days.

The fact that the ultrafiltration was carried out at 0-2°C, and that toluene was added, was undoubtedly responsible to a large extent for the prevention of growth of these bacteria during preparation of the tuberculin, although these precautions apparently were not sufficiently effective to prevent contamination.

As mentioned later, all the individual lots were tested for biological activity by intracutaneous injection into guinea-pigs. It was found that the contamination had no effect on the activity of the preparations concerned.

Yield and biological activity of individual lots of PPD

Variations in yield. Details of the yield of PPD, and other experimental data for each lot, are shown in the accompanying table.

EXPERIMENTAL DATA CONCERNING 95 LOTS OF PURIFIED TUBERCULIN

Lot number	Strain	Volume of medium (litres)	pH of culture filtrate	Purified tuberculin		
				Yield		Biological activity *
				total (g)	relative (mg/litre)	
1	E 9656	148	5.6	4.60	30	–0.7
2	E 5	147	5.6	5.26	35	–0.3
3	,,	150	6.4	16.81	110	0.5
4	,,	150	6.5	18.64	125	0.7
5	,,	154	6.0	19.53	125	0.9
6	,,	150	6.1	14.08	95	0.2
7	,,	150	6.7	17.13	115	0.3
8	,,	139	6.7	20.20	145	–0.7
9	,,	124	6.6	15.42	125	1.5
10	,,	150	6.4	12.45	85	–0.3
11	,,	149	6.4	16.04	110	1.2
12	,,	124	6.3	10.56	85	–1.2
13	E 9656	150	5.7	10.10	65	0.9
14**	,,	101	5.8	6.84	70	0.0
15	,,	100	5.8	9.86	100	0.2
16**	,,	100	5.9	9.76	100	–0.5
17	,,	100	6.6	8.41	85	–0.4
18	,,	100	7.0	13.00	130	0.1
19	,,	100	6.8	8.64	85	0.8
20	,,	100	7.0	12.14	120	0.0
21	,,	50	6.7	7.11	140	–0.2
22	,,	95	6.6	14.53	155	–1.0
23	,,	90	6.4	11.90	130	–1.1
24	,,	106	6.5	13.31	125	–0.9
25**	,,	107	6.3	10.35	95	–0.2
26**	,,	108	6.2	10.23	95	–0.9
27	T 3487	93	8.3	12.35	130	0.0
28	,,	95	8.3	6.27	65	–0.2
29	,,	98	8.2	8.91	90	2.2
30	,,	99	7.4	12.03	120	0.1
31	,,	98	7.0	8.13	80	0.5
32	,,	97	6.8	9.80	100	0.9
33	,,	98	7.4	10.79	110	1.2
34	,,	98	7.3	9.14	90	0.4
35	,,	100	7.2	12.71	125	–0.2
36	,,	97	7.8	9.70	100	0.9
37	,,	100	7.3	7.83	80	1.0
38	,,	106	6.7	10.55	100	0.5
39	U 1921	103	6.8	7.10	70	0.4
40	,,	100	7.4	6.85	70	–0.1
41	,,	95	8.1	11.99	125	0.0
42	,,	96	7.3	8.67	90	2.1
43	,,	96	7.3	7.85	80	–0.2
44	,,	108	7.5	10.60	100	0.3
45	,,	108	7.5	11.31	105	0.2
46	,,	100	6.1	8.67	85	0.0
47	,,	100	6.5	9.97	100	0.2
48	,,	98	6.3	8.39	85	–0.4
49	,,	46	7.4	6.22	135	1.5
50**	,,	93	7.4	7.80	85	0.5

EXPERIMENTAL DATA CONCERNING 95 LOTS OF PURIFIED TUBERCULIN
(concluded)

Lot number	Strain	Volume of medium (litres)	pH of culture filtrate	Purified tuberculin		
				Yield		Biological activity *
				total (g)	relative (mg/litre)	
51	T 3419	100	5.9	8.16	80	0.7
52	,,	100	5.8	9.42	95	0.1
53	,,	100	5.8	5.25	55	0.6
54	,,	100	5.8	6.30	65	–0.6
55	,,	100	6.6	6.06	60	0.1
56	,,	100	6.1	5.61	55	–0.1
57	,,	100	6.0	4.36	45	–0.1
58	,,	110	6.1	5.38	50	0.0
59**	,,	93	5.9	5.67	60	–0.2
60**	,,	93	5.9	7.76	85	–1.0
61**	T 3480	100	5.7	5.61	55	0.0
62	,,	100	5.7	3.87	40	–1.7
63	T 3419+T 3480	110	5.9	3.56	30	0.9
64**	T 3480	100	5.8	4.11	40	–0.7
65**	,,	100	5.8	8.73	85	–0.8
66	,,	100	5.6	3.85	40	–1.9
67**	,,	100	5.6	3.38	35	–1.1
68	,,	95	5.7	2.63	30	0.5
69	T 3487	100	7.2	7.10	70	–0.4
70	,,	92	7.2	7.03	75	–0.4
71	,,	85	7.3	8.02	95	–0.1
72	,,	100	7.3	7.74	75	–0.6
73	,,	100	7.3	9.74	95	0.2
74	E 1580	100	8.3	8.50	85	0.0
75	,,	100	8.3	10.17	100	–1.4
76	,,	98	7.4	8.26	85	0.4
77	,,	98	7.4	10.60	110	0.3
78**	,,	98	7.0	10.02	100	–0.9
79**	,,	98	7.0	9.61	100	–1.1
80**	,,	88	7.9	6.03	70	0.1
81	,,	88	7.9	4.70	55	0.0
82**	,,	100	6.1	5.45	55	–0.1
83**	,,	100	6.1	6.52	65	0.6
84**	,,	105	7.3	7.80	75	–0.3
85**	,,	105	7.3	7.27	70	–0.6
86	,,	95	8.2	12.24	130	–1.2
87	,,	95	8.2	8.84	95	–0.2
88	,,	100	8.6	10.50	105	–0.7
89	,,	100	8.6	11.38	115	0.7
90	,,	97	8.0	10.58	110	0.5
91	,,	97	8.0	8.50	90	–0.2
92	,,	100	8.5	9.85	100	–0.4
93	,,	100	8.5	7.14	70	–0.6
94	,,	100	8.4	9.08	90	–0.4
95	,,	100	8.4	7.31	75	1.0

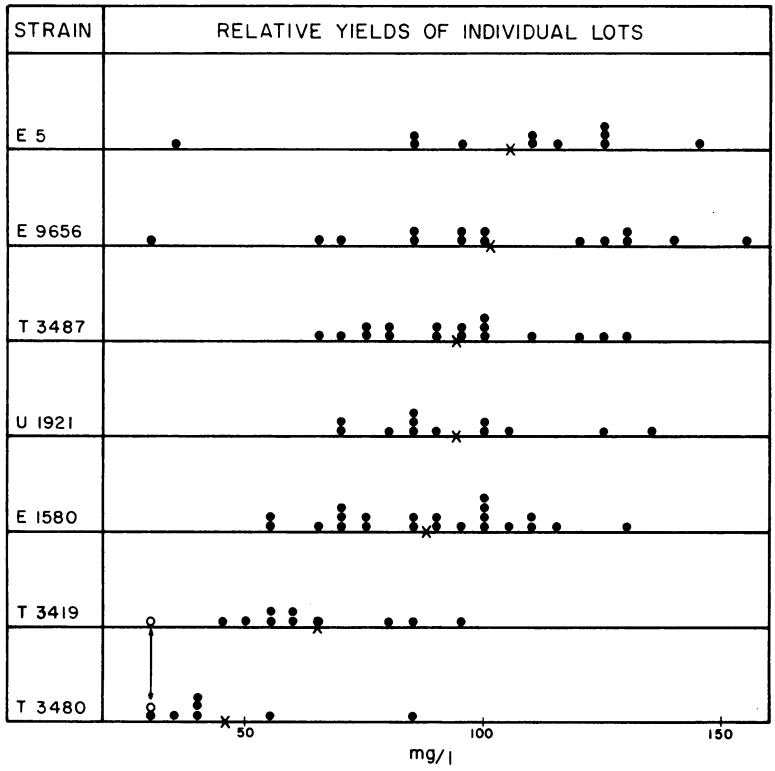
*** The deviation (in mm) of the reaction from the average for all 95 lots (9.4 mm). Each mean is based on 6 tests with each lot. The standard error of the means is 0.55 mm.**
**** Not included in RT 23**

It will be seen that the relative yield of PPD varies considerably from one lot to another: from 30 mg per litre in lots 1, 63 and 68 to 155 mg per litre in lot 22.

These variations in yield may be due either to variations in the loss of PPD during preparation or to variations in the amount of tuberculin present in the culture filtrates used for the various lots.

PPD is composed mostly of protein, and therefore it is possible to calculate approximately the loss of PPD during preparation by measuring the protein content at various stages.[1] It has been calculated from protein analyses that, on the average, about 35-40% of the PPD was lost during

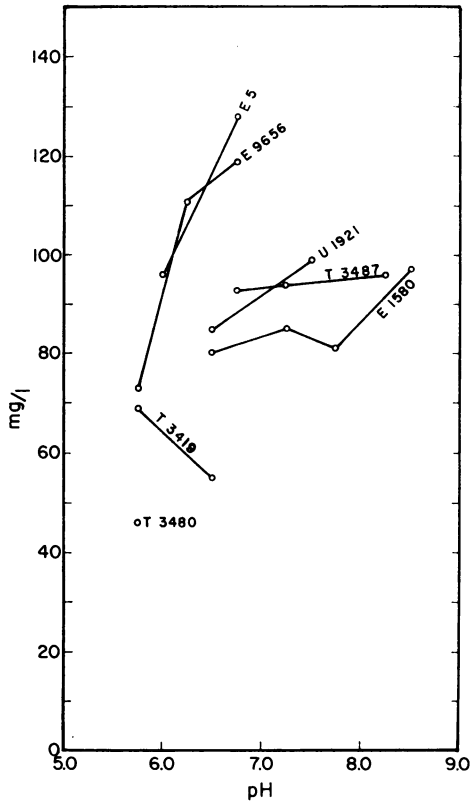
FIG. 1. RELATIVE YIELDS (IN mg PER LITRE OF MEDIUM) OF INDIVIDUAL LOTS OF PURIFIED TUBERCULIN OBTAINED FROM DIFFERENT STRAINS OF TUBERCLE BACILLI



The average yield for each strain is indicated by x. The strains have been arranged according to decreasing average yield. One lot, No. 63, was prepared from the culture filtrates of two strains (T 3480 and T 3419).

[1] The determination of protein was made by means of the biuret reaction, after precipitation of the protein with 5% trichloracetic acid, using the Weichselbaum (1946) biuret reagent. The results are not given in detail in this paper.

FIG. 2. RELATIVE YIELDS OF PURIFIED TUBERCULIN ACCORDING TO pH OF CULTURE FILTRATE, MEASURED AFTER HEAT STERILIZATION OF THE BACTERIAL CULTURES



preparation. The loss was greatest during the sterile filtration (30%), the concentration, precipitation and dehydration accounting for only 5-10%. The loss during filtration was due partly to adsorption (15%) and partly to the fact that a constant volume of culture filtrate remained in the Seitz and Berkefeld filters after filtration (15%).

Since the loss of PPD was found to be fairly constant for the various lots, the varying yields for the different lots are probably due to variations in the protein content of the culture filtrates.

The variations in the yield observed with the different strains used for production will be seen from Fig. 1. Strains T 3480 and T 3419 gave smaller yields than the other strains.

The variations in yield were also considerable *within* the strains. This is most evident in the case of E 9656. In some instances the yield seems to be related to the pH of the sterilized culture filtrates,[1] as will be seen from Fig. 2. As regards strains E 9656 and E 5, the yield decreases with decreasing pH, and the low yields obtained with T 3480 are probably due to the low pH values of all the culture filtrates from this strain. With the other strains, however, the variations in the pH of the culture filtrates seem to have had less effect on the size of the yield.

Biological assay of individual lots. In order to be able to exclude any inactive lots from the final preparation and to obtain an estimate of the variations in the potency of the individual lots, a biological assay was

[1] The pH was measured electrometrically, by means of a Radiometer pH meter 22, in about 10% of the sterilized culture. The values shown in the table on pages 833 and 834 are averages for each filtration.

made on all the 95 lots of tuberculin by means of intracutaneous tests on BCG-vaccinated guinea-pigs.

Experimental animals. Sixty white guinea-pigs, vaccinated intracutaneously with four injections of BCG vaccine (BCG Department, Statens Seruminstitut), were used for the assay three to five months after vaccination. In order to increase the tuberculin sensitivity of some of the animals, 40 of them were revaccinated intracutaneously with 0.1 ml of BCG vaccine (BCG Department, Statens Seruminstitut) two weeks before the assay.

Tuberculin dilutions. Dilutions containing 0.1 μg of PPD per 0.1 ml (supposedly about 5 TU per 0.1 ml) were prepared from all the 95 lots. For reference purposes, tuberculin dilutions from the previous batch of tuberculin, RT 22, containing 0.013 μg, 0.067 μg and 0.33 μg of PPD (1 TU, 5 TU and 25 TU) per 0.1 ml, were also included in the assay. The diluent used was phosphate-buffered saline of pH 7.38, containing 0.1‰ Chinosol as preservative.

Experimental design. Each animal was tested with ten different tuberculin preparations, distributed at random among the test-sites. The tests with the various tuberculins were allocated to the animals according to three double 10×10 lattice designs (Cochran & Cox, 1955).

The assay was carried out over three days, 20 animals being injected each day.

Reading. The reactions were read after 24 and 48 hours, each time by two readers. The longitudinal and transverse diameters of erythema were recorded in mm.

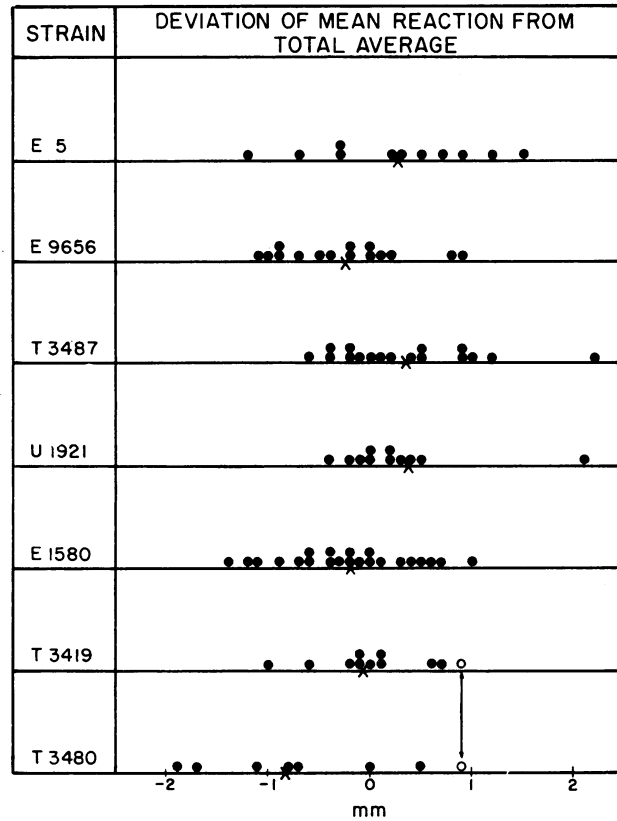
The data presented in the table on pages 833 and 834 are based on the averages of the readings after 24 hours, and the results have been corrected for variations due to the different degrees of tuberculin sensitivity of the guinea-pigs. The results obtained after 48 hours were similar. The figures shown in the table represent the difference (in mm) between the reaction evoked by the individual lot and the average for all 95 lots (9.4 mm). The standard deviation of the means is 0.55 mm.

It will be seen that some lots (e.g., 29 and 42) are significantly stronger and others (e.g., 62 and 66) significantly weaker than the average. The differences in average reactions correspond roughly to the stronger lots being about five to ten times stronger than the weaker lots.

In order to test the reproducibility of these findings, two further assays were made with the two lots which gave the strongest reactions (29 and 42) and with the two which gave the weakest reactions (62 and 66). Significant differences were found between the strong and the weak lots in these experiments also.[1] However, as might be expected on the assumption

[1] The results are not included in this report.

FIG. 3. BIOLOGICAL ACTIVITY OF INDIVIDUAL LOTS OF PURIFIED TUBERCULIN OBTAINED FROM DIFFERENT STRAINS OF TUBERCLE BACILLI, ASCERTAINED BY INTRADERMAL TESTS IN BCG-VACCINATED GUINEA-PIGS



The average value for each strain is indicated by x. One lot, No. 63, was prepared from the culture filtrates of two strains (T 3480 and T 3419).

that the deviations of the average reactions were overestimated in the main assay for the stronger and weaker lots, owing to errors of random sampling, the differences were somewhat smaller in these two assays.

The variations in the biological activity of the various lots are due partly to the use of different strains in the preparation. The biological activity of the lots prepared from each strain is shown in Fig. 3. The PPD obtained from strain T 3480 appears to be weaker that that obtained from the other strains. An analysis of variances showed that about half the variations between the lots can be attributed to variations between strains. The PPD from strain T 3480 was significantly weaker than the PPD obtained

from the other strains. No significant variations were found between the other strains.

Attempts to find other factors responsible for the variations in biological activity were fruitless. Consideration was given to the possibility that the variations were correlated with the varying yields of PPD, and were largely due to differences in the extent of co-precipitation of inactive impurities. However, no correlation was found between the yield and the biological activity of the individual lots, nor between the biological activity and the pH of the culture filtrates.

Even though significant differences in the potency of the various lots were found in these assays, none of the lots was so weak as to justify its exclusion from the final mixture.

Selection of lots to be included in RT 23

As mentioned above, no justification was found for excluding any of the 95 lots on account of low biological activity. However, some of the lots were, in fact, excluded from the final preparation on other grounds—namely, because a precipitate was found in stock solutions prepared from them.

Precipitate with Chinosol in stock solutions. As the first step in the preparation of dilutions of purified tuberculin, a stock solution containing 1 mg of PPD per ml is generally prepared. This stock solution is then diluted further to the desired concentration with a suitable diluent. The stock solution and dilutions contain $0.1^0/_{00}$ Chinosol as preservative.

Jensen et al. (1938) had observed previously that, after being stored for some time, certain stock solutions became darker or turbid, and sometimes a dark precipitate was formed,owing to the formation of insoluble complexes between the Chinosol and iron impurities. In some of the stock solutions prepared from the present 95 lots, such a precipitate, varying in extent, was also observed after some time.

Exclusion of lots. In order to avoid precipitation in the stock solutions, it was decided to exclude from the final preparation the lots (18 in all) in which precipitation was most evident. The lots excluded are indicated by asterisks (**) in the table on pages 833 and 834.

A similar procedure had been adopted previously, in the preparation of RT 22.

Preparation of RT 23

It would be very difficult to ensure sufficiently good mixing if the single lots were mixed in powder form. It was decided, therefore, to mix the individual lots in suspension in ether.

In order to ascertain whether this procedure would bring about any changes in the qualities of the preparation, a pilot batch, RT 23p, was mixed in the way planned for RT 23, and biological assays in guinea-pigs and humans were carried out with the mixture. These assays (unpublished observations) showed that the biological properties of RT 23p were not affected by the procedure used for the mixing, and the final mixing was therefore carried out as planned. Before mixing, however, small samples (100-300 mg) were taken from each of the 77 lots to be included in RT 23, in order to have some material available for possible later tests on the individual lots.

The remainder of the 77 lots (about 700 g in all) was poured into a large Erlenmeyer flask containing anhydrous ether. The flask was shaken carefully to ensure adequate mixing of the contents. The mixed preparation was then centrifuged in large steel beakers and the ether allowed to evaporate. The few lumps present were crushed by stirring with glass rods, and the preparation was finally filled into smaller Erlenmeyer flasks or glass tubes, to be stored at room temperature. The final yield was 670.5 g.

Some very hard lumps in the large Erlenmeyer flask were not included in batch RT 23. The weight of this residue was 27.9 g.

Discussion

In the preparation of the individual lots, as described above, variations in yield and biological activity were noted. Similar variations had been observed previously (Jensen et al., 1938; Lind, 1948).

The variations in yield are due mainly to variations in the tuberculin content of the culture filtrates used for the production, and to a lesser extent to variations in the loss of tuberculin during preparation.

Some of the variations in the tuberculin content of the culture filtrates are due to the use of different strains for the production, since the average yield of PPD varies for the different strains. Lind (1948) assumed that the ability of strains of tubercle bacilli to form tuberculin varied and was occasionally lost after several subcultures *in vitro*. The present observations do not support the latter assumption, since the higher yields were obtained with old laboratory strains which had been subcultured *in vitro* for more than ten years.

The differences in the tuberculin yield with different strains may be due to basic differences in the ability of the strains to produce tuberculin, or may be explained by differences in the growth rate of the various strains. The latter explanation is the most probable. The strains used have been found in other experiments to differ in their rates of growth, and since a constant and fairly short incubation period (5 weeks) was used for all the strains, some of the cultures may not have been fully grown at the time

of harvesting. However, variations in yield have occurred with the fully grown cultures also, partly for the following reason. During the growth of human strains of tubercle bacilli on Sauton medium, the pH of the culture filtrate will first increase to 8-8.5 and then decrease to 5-6. At these lower pH values the tuberculo-protein is adsorbed to some extent or is otherwise bound to the bacterial cells (McIntosh & Konst, 1943, 1949; Svenkerud, 1955; Wong, 1937) and thus the actual tuberculin yield of old acid cultures may be small.

Even though the relationship between the pH and the tuberculo-protein content of the culture filtrate is not the same for different strains, as observed also by Lind (1948), there is no doubt that an increased yield could have been obtained in some of the acid culture filtrates by neutralizing the culture filtrate with sodium hydroxide before heat sterilization of the fully grown cultures. However, such a procedure was not used in the preparation of RT 23, since it would have involved a change in the production methods.[1]

With a few exceptions, the variations in biological activity of the individual lots are small. Some of these are due to one strain—T 3480—having given weak preparations. No significant differences in biological activity were found between the six other strains, nor could any other causes be found for the small variations observed.

It is generally assumed that the biological activity of PPD is due to the protein content (McIntosh & Konst, 1949; Seibert, 1950), and it has almost become a standard practice to adjust different preparations of PPD to the same protein concentration before use, or before a biological standardization is carried out (Edwards & Krohn, 1957; Green, 1946). Among the present 95 lots, the two weakest were characterized by low protein content (unpublished observations), otherwise, no correlation could be found between the biological activity and the protein content of the individual lots.

Serological tests carried out on the final product have shown that, as in the case of RT 22, RT 23 is a useful antigen in the haemagglutination reaction,[2] both by the Middlebrook & Dubos (1948) test and by Boyden's tannic acid test (Boyden, 1951).

As regards the suitability of RT 23 for use in large-scale field tuberculin testing under widely varying experimental conditions, no definite basis for evaluation of this aspect as yet exists. However, some knowledge of the properties of the new preparation when used for skin testing have already been obtained from the comprehensive biological assays performed.[3] In addition, the procedures chosen for production should ensure some

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[1] The neutralization procedure has been used subsequently, with satisfactory results, for the preparation of purified tuberculin from various types of acid-fast bacteria.

[2] H. C. Engbæk, Tuberculosis Department, Statens Seruminstitut: personal communication

[3] See article on page 845 of this number of the *Bulletin*.

justification for presuming that the preparation is suitable, since other batches—RT 19-21 and RT 22—produced by the same method have been used extensively and proved satisfactory.

RÉSUMÉ

Les diverses préparations de tuberculine utilisées pour les tests intradermiques chez l'homme présentent souvent des différences d'activité et de spécificité qui invalident la comparaison des résultats. Afin d'éliminer certaines causes d'erreur, un nouveau lot de tuberculine purifiée (PPD) a été préparé à la demande du FISE. Ce lot de 670 g doit suffire aux besoins des campagnes de vaccination pendant un certain nombre d'années. La préparation de ce nouveau lot, RT 23, est décrite dans cet article.

On a mélangé 95 lots de tuberculine purifiée, préparée selon la même méthode et, dans la mesure du possible, selon la technique adoptée par le Statens Seruminstitut pour les lots précédents, RT 19-21, RT 22, employés avec succès pour les épreuves tuberculiniques des campagnes de vaccination.

Chaque lot a été élaboré comme suit : environ 100 flacons d'un litre de milieu de Sauton légèrement modifié ont été ensemencés de bacilles tuberculeux humains et mis à l'étuve à 38°C pendant 5 semaines. Les cultures ont alors été stérilisées par la vapeur pendant 1 heure, puis filtrées sur une gaze, puis sur un filtre Seitz grossier et enfin sur bougies Berkefeld. Le filtrat stérile a été ensuite concentré par ultrafiltration sur collodion. Puis la tuberculine a été précipitée par l'acide trichloracétique, à la concentration finale de 2 %. La tuberculine a été deshydratée par agitation du précipité à plusieurs reprises dans l'éther anhydre.

Afin de compenser d'éventuelles différences de spécificité, sept souches de bacilles tuberculeux humains ont été utilisées pour la préparation du lot RT 23. Quatre d'entre elles étaient d'anciennes souches de laboratoire, trois avaient été récemment isolées de malades.

Pour chacun des 95 lots, on indiquait: la souche de bacilles utilisée, le pH moyen du filtrat de la culture, le rendement et l'activité biologique relative, déterminée, par voie intradermique, sur des cobayes vaccinés au BCG.

Le rendement des lots a varié entre 30 et 155 mg/l de milieu. Ces variations s'expliquent en partie par l'emploi de plusieurs souches de bacilles pour un même lot, le rendement individuel des souches étant lui-même variable. Dans deux souches, le rendement a diminué avec le pH du filtrat.

L'activité variait aussi d'un lot à l'autre. Certaines souches donnaient une tuberculine nettement plus faible. Aucun lot cependant n'a été assez peu actif pour qu'on l'écarte. Dans quelques-unes des solutions-mères, préparées à partir de chaque lot et contenant 1 mg de tuberculine purifiée par ml, des précipités foncés ont apparu, dus à la formation de complexes insolubles entre les impuretés ferriques et le Chinosol employé comme agent conservateur des dilutions de tuberculine. Dix-huit lots présentant des précipités trop importants ont été éliminés. Les 77 lots restants ont été mis en suspension dans l'éther anhydre, mélangés et centrifugés pour constituer la préparation finale RT 23.

Des tests de stérilité ont été effectués soit sur la préparation définitive, soit sur les lots individuels. La préparation finale RT 23 était stérile. La contamination occasionnelle de certains lots a été observée, après 4-10 jours d'incubation de la culture de contrôle. Mais elle n'avait apparemment aucune action sur l'activité de la préparation.

REFERENCES

Boyden, S. V. (1951) *J. exp. Med.*, **93**, 107
Cochran, W. G. & Cox, G. M. (1955) *Experimental designs*, New York, p. 280
Edwards, L. B. & Krohn, E. F. (1957) *Amer. J. Hyg.*, **66**, 253
Green, H. H. (1946) *Vet. J.*, **102**, 267
Jensen, K. A., Bindslev G., Møller, S., Hansen, A. & Lind, P. (1938) *Tubercle (Lond.)*, **19**, 433
Lind, P. (1947) *Acta tuberc. scand.*, **21**, 111
Lind, P. (1948) *Acta tuberc. scand.*, **22**, 287
McIntosh, C. W. & Konst, H. (1943) *Canad. J. publ. Hlth*, **34**, 557
McIntosh, C. W. & Konst, H. (1949) *Canad. J. comp. Med.*, **13**, 173
Middlebrook, G. & Dubos, R. (1948) *J. exp. Med.*, **88**, 521
Seibert, F. B. (1950) *Schweiz. Z. Tuberk.*, No. 3, p. 1
Svenkerud, R. (1955) *A study of heat concentrated synthetic medium tuberculin*, Copenhagen (Thesis)
Weichselbaum, T. E. (1946) *Amer. J. clin. Path.*, **10**, 40
Wong, S. C. (1937) *J. Bact.*, 33, 451