LABORATORY AND CLINICAL INVESTIGATIONS ON TUBERCULIN PURIFIED PROTEIN DERIVATIVE (P.P.D.) AND OLD TUBERCULIN (O.T.)

BY

A. T. DOIG, M.D., D.P.H.*Medical Superintendent, Oakwood Hall Sanatorium, Rotherham***G. GEMMILL, M.B., D.P.H.***Deputy Medical Officer of Health, Lincoln***G. GREGORY KAYNE, M.D., M.R.C.P., D.P.H.***Deputy Medical Superintendent, County Sanatorium, Clare Hall; late Dorothy Temple Cross Research Fellow in Tuberculosis***F. V. LINGGOOD, Ph.D., B.Sc.***Wellcome Physiological Research Laboratories, Beckenham***H. J. PARISH, M.D., M.R.C.P.Ed., D.P.H.***Wellcome Physiological Research Laboratories, Beckenham*

AND

J. S. WESTWATER, M.D., D.P.H.*Late Senior Assistant Medical Officer, Hospitals Service, London County Council*

The value of the Mantoux test as a clinical aid seems to be doubted in some quarters, apparently on the assumption that there is almost complete tuberculinization of the population. This may still be true of crowded urban districts, but recent epidemiological surveys have shown that in many communities a large percentage of people reach adolescence without having been infected, and, moreover, that this percentage is becoming greater as the incidence of tuberculous disease is decreasing. There can be little doubt, therefore, that with time the Mantoux test will be found more and more useful in clinical work.

Since the Mantoux test is a quantitative one, the material used in its performance should be stable, and constant in composition and potency; further, the exact amount of the active principle injected should be known. Only if these conditions are fulfilled will it be possible to compare repeated tests at intervals on the same patient, and to correlate or pool the observations of different workers investigating large groups. Until a few years ago Koch's old tuberculin was always used; but it was increasingly realized that this crude material did not fulfil the conditions laid down above.

Old tuberculin is usually prepared by the growth of the tubercle bacillus on a veal-infusion-peptone medium containing a comparatively large amount of glycerol. The mature culture is steamed and the filtrate evaporated to one-tenth of its original volume. Thus it is a complex mixture, containing, besides the active principle, much non-specific material, such as peptones, salts, glycerol, and bacillary metabolic products. Long (1934) has stated that its potency is due to about 1 per cent. of its total substance. It has been found that batches of old tuberculin prepared not only from different strains, but even from the same strain and in the same type of culture medium, may vary in potency. Moreover, no accurate method of standardization has been developed. The "shock method," which was found to carry a large error, was replaced by intracutaneous testing in guinea-pigs, but even this may allow a 40 per cent. error unless a large number of comparisons are made (Okell and Parish, 1927). Finally, the use of old tuber-

culin has the disadvantage that dilutions deteriorate comparatively rapidly.

These considerations have stimulated much research work, particularly in America, to eliminate non-specific factors and thus prepare a purer product. The first important step was made when Long and Seibert (1926) showed that the tubercle bacillus would grow and produce a potent tuberculin on a synthetic medium with asparagine and ammonium salts as the sole sources of nitrogen. It was found that protein-like substances were formed during growth on this medium, and this led to the view that the active principle was of a protein nature, as suggested much earlier by Koch. Definite confirmation was obtained by Seibert (1928), who prepared unheated culture filtrates of the organism grown on Long's medium, and was able to isolate a crystalline protein which is water-soluble and a very active tuberculin. Repeated recrystallization of this product increased rather than decreased its potency, demonstrating conclusively the protein nature of the active principle. This method is impracticable for the routine production of a pure tuberculin for the following reasons: (1) the amount of tuberculo-protein which will crystallize is very small; (2) the protein which will not crystallize is also physiologically active; (3) when the product is injected there is some risk of producing sensitization which might lead to errors in reading. In this connexion Seibert (1933) has shown that it is possible to isolate the active principle in different states of molecular aggregation according to the method of preparation. Potent tuberculin preparations, all of a protein nature, have been made with different molecular weights—namely: (a) ammonium sulphate fraction from unheated culture filtrates (Seibert's TPA)—molecular weight = 25,000; (b) trichloroacetic acid precipitate from unheated culture filtrates (Seibert's TPT)—molecular weight = 3,000 to 8,000; and (c) trichloroacetic acid precipitate from steamed and evaporated culture filtrates (Seibert's SOTT—"synthetic medium old tuberculin trichloroacetic-acid-precipitated")—molecular weight = 2,000. The sensitizing power of these preparations has been investigated; with the exception of SOTT which possesses the lowest molecular weight, they are capable of eliciting reactions of the Arthus type when injected repeatedly into the skin of guinea-pigs and of producing precipitins in rabbits. Obviously it is undesirable to use preparations with which there is the slightest risk of sensitization, for they may lead to erroneous results (Aronson and Nicholas, 1933); the best diagnostic agent so far produced is therefore the non-sensitizing SOTT, which Seibert (1934) has more recently named "purified protein derivative." This is simply the substance of the lowest molecular weight which will cause the tuberculin reaction in sensitive subjects. It is isolated as a dry powder, any desired doses of which can be issued to doctors in sterile form in bottles or in the shape of tablets. Solutions are made up very conveniently as required. It is claimed that P.P.D. is always of the same composition and potency. Variation in strain of bacillus or luxuriance of growth merely causes variation in yield of P.P.D. Thus an exact known quantity by weight of the effective substance is always injected. The other advantages claimed are absence of non-specific substances, increased stability, and the convenient preparation of dilutions.

I. LABORATORY INVESTIGATIONS

By F. V. LINGGOOD and H. J. PARISH

In this section we propose to deal with the preparation, potency, and stability of purified protein derivative (P.P.D.) and of "dried dilutions" of P.P.D.

Preparation of Purified Protein Derivative

We have adhered closely to Seibert's method (1934). The bacillus is grown on a synthetic protein-free medium, from which an old tuberculin is prepared by steaming and evaporation. The bulk of the impurities is removed by ultrafiltration and the active principle precipitated with trichloroacetic acid. The precipitate is thoroughly washed with this reagent, and the acid is then removed with ether, which at the same time dehydrates the protein derivative.

For those who are specially interested in the method of preparation, technical details are as follows:

A suitable strain of tubercle bacillus is grown as a film in large 20-litre ("rose-water") bottles on the synthetic medium of Long and Seibert (1926), which in our hands has given a better product than certain other synthetic media. After two to three months the mature cultures are killed by steaming for two hours, and the organisms are then removed by filtration through paper pulp. Glycerol is added in the proportion of 122 c.cm. to each litre of original culture fluid, and the mixture is evaporated on a steam bath to one-fifth to one-tenth of its original volume. This concentrate is diluted with an equal volume of 1 per cent. phenol in distilled water, left in the cold room for some days for any precipitate to collect, and then filtered warm through a Berkefeld candle. By this means a synthetic medium old tuberculin is produced which has now to be freed from all extraneous matter.

A preliminary purification is made by ultrafiltration, using the technique described by Quigley (1934), except that a special collodion is used (I.C.I. HX45 as a 13 per cent. solution in glacial acetic acid).

The synthetic medium old tuberculin prepared as above is diluted with an equal volume of 0.5 per cent. phenol water, and drawn through the ultrafilters. The active principle remains outside the filters, while salts, glycerol, amino-acids, and other non-colloidal constituents of the crude tuberculin pass through the membranes. Washing with 0.5 per cent. phenol water is continued until the ultrafiltrate is practically chloride-free.

The solution outside the membrane, concentrated to a small volume, consists mainly of the active principle bound to polysaccharide. The latter is removed by the addition of one-quarter volume of freshly prepared 50 per cent. trichloroacetic acid. The resulting precipitate, which consists almost entirely of the pure protein derivative (Seibert, 1932), is collected by centrifugalization, and washed six to eight times with fresh 10 per cent. trichloroacetic acid to remove the contaminating polysaccharide.

After the final washing the precipitate is smeared rather thickly inside the centrifuge tube, and the excess of water is removed by drying *in vacuo* for half an hour, but complete drying must be avoided. (From this stage onwards great care must be taken to prevent any of the powder being inhaled by the manipulator. All operations are conducted inside a box with a closely fitting glass lid and "sleeves" to protect the arms of the manipulator. Suction from a water-pump is gently applied to one side of the box to prevent any of the powder diffusing around the lid into the air of the room. As a further precaution rubber gloves and a respirator should be worn.) Large volumes of dry ether are used to extract the water and trichloroacetic acid from the precipitate by alternate trituration and decantation through a Soxhlet thimble; the process is repeated until the precipitate is in the form of a very fine powder. By this time it will be found that the ether coming through the Soxhlet thimble gives no acid reaction. The fine powder is transferred to the thimble and is thoroughly washed again with dry ether. The thimble is then placed over paraffin wax in a desiccator, and the last traces of ether removed *in vacuo*. As tuberculin adheres tenaciously to filter-candles and glassware (Parish and O'Brien, 1935), the apparatus in the tuberculin laboratory must on no account be employed for other biological work.

Potency and Stability of Purified Protein Derivative

The dry purified protein derivative is a tan-coloured powder. Although minor variations have been noticed occasionally, the potency of different preparations has been remarkably constant. When redissolved at a concentration of 5 mg. per c.cm., successive batches almost invariably have been equal in potency to the international standard old tuberculin on guinea-pig intracutaneous tests. The solutions for these tests are prepared by the addition of a small amount of N/10 alkali to the powder in order to dissolve it; borate buffer solution is added subsequently and the pH adjusted to 7.4, the final concentration being 5 mg. per c.cm. The dry purified protein derivative is a very stable preparation.

"Dried Dilutions" of Purified Protein Derivative: Preparation, Potency, and Stability

In America the active principle is incorporated in tablets with a dry diluent, beta-lactose (Reichel and Clark, 1934), and issued in two strengths. We have adopted a somewhat different procedure. The dry powder is weighed and dissolved as described above to give a final strength of 5 mg. per c.cm. The potency of the material, after filtration through a Berkefeld candle, is checked on tuberculous guinea-pigs on a number of occasions to ensure that it is equivalent to international standard old tuberculin. The standardized solution of P.P.D. is then diluted under sterile conditions in a special boric-acid-borate buffer solution of pH 7, the final dilutions being distributed in bottles which contain 1 c.cm. (ten doses of 0.1 c.cm.) of strengths equivalent to 1 in 10,000, 1 in 1,000, and 1 in 100 of international standard old tuberculin. These dilutions are evaporated to dryness *in vacuo* under sterile conditions. Immediately before use 1 c.cm. of a special borate diluent is added to the required bottle, which is left stoppered for two minutes for the "dried dilution" to dissolve, and is then well shaken. The strength of the boric acid, borate, and salt in the final diluent is adjusted so that the final mixture is isotonic and of pH 7.5. The dose of the reconstructed "dried dilution" is 0.1 c.cm. The actual weights of P.P.D. prepared for injection are 0.00005 mg. (that is, 0.1 c.cm. of dilution 1 in 10,000), 0.0005 mg., and 0.005 mg.

Before issue from the laboratories the potency of each batch of "dried dilutions" is tested on tuberculous guinea-pigs against freshly prepared dilutions of international standard old tuberculin. For these tests the "dried dilutions" are redissolved in the special diluent, and may then be diluted further in physiological saline to provide a suitable range of dilutions for the injections. Thus a dilution of 1 in 500 is generally prepared from the 1 in 100 "dried dilution," and dilutions of 1 in 2,000 and 1 in 4,000 from the 1 in 1,000 "dried dilution." Checking the potency of the 1 in 10,000 "dried dilution" is only practicable on very sensitive guinea-pigs. So far we have been unable to detect any loss in potency during preparation or after storage for three years at room temperature. After re-solution, however, deterioration is more rapid than that of corresponding dilutions of old tuberculin.

With reference to the particular "dried dilutions" of P.P.D. which were used in the following clinical study, our aim was to make them as nearly equal to the corresponding dilutions of old tuberculin as possible. Actually they proved to be very slightly stronger, although this difference was apparent only after repeated tests on the

skin of very sensitive guinea-pigs. Altogether forty-six guinea-pigs have now been used, with the following results:

P.P.D. = O.T. on 12 guinea-pigs
 " > " " 22 "
 " < " " 12 "

We would emphasize that the difference between the dilutions of the two preparations was not detectable on all the guinea-pigs, and that only our most sensitive animals indicated that the dilutions of P.P.D. were a trifle stronger. Twofold differences were clearly distinguishable on the whole of the guinea-pigs, and it was agreed by all who observed the reactions that the difference in potency indicated in the above table was very much less than twofold.

II. CLINICAL INVESTIGATIONS:

(a) Mantoux Tests: A Comparison of Purified Protein Derivative with Old Tuberculin

The present investigations were undertaken to compare P.P.D. with old tuberculin in clinical work. At first sight this is absurd, since we are apparently comparing two substances made comparable by standardization. But since the standardization is carried out in guinea-pigs, such an investigation is still indicated in man, and might serve to emphasize any deficiencies of the guinea-pig standardization. Preliminary tests on human subjects made previously by Davis and Guzzard (1935) and Westwater (unpublished) have shown that "wet" dilutions of P.P.D. deteriorated more rapidly than corresponding dilutions of old tuberculin and were thus unsuitable for use. Therefore, as mentioned earlier in this paper, "dried dilutions" of P.P.D. and also a suitable borate buffer diluent were supplied to us from the Wellcome Laboratories. Corresponding dilutions of old tuberculin in 0.5 per cent. phenol saline were also provided, and, on account of the possibility of deterioration, were used within a fortnight (very occasionally three weeks) of receipt.

Particular care was taken in performing the tests and in recording the results. The usual technique was followed—that of injecting 0.1 c.cm. intradermally, choosing comparable sites on each forearm; and, in view of the extreme "tenacity" of tuberculin to glass (Parish and O'Brien, 1935), separate syringes were reserved, and employed, for each type of tuberculin and for each individual dilution. Some of us found that for accurate work it was helpful to have only one dose in the syringe at a time—that is, the tuberculin drawn up to the 0.1 c.cm. mark—so that attention could be concentrated on raising a proper intradermal "wheal."

The reaction to the intradermal injection of 0.1 c.cm. of tuberculin in a sensitized person is an erythema, an erythematous infiltration, or an actual oedema of the skin, usually at a maximum after forty-eight hours and varying in size. Mild reactions may present difficulty in interpretation, and there is no universal agreement on what constitutes the minimum reaction to be accepted as positive. Different authorities have taken as suitable criteria areas of erythema varying in diameter from 2 mm. to 10 mm., with or without associated infiltration. In addition there is considerable difference of opinion concerning the best time to read results, but most workers agree that a twenty-four-hour reading is not reliable. Hart (1932) accepts as a positive reaction an erythema or erythematous infiltration whose greatest diameter is 5 mm. or more, taking the forty-eight-hour reading; and Aronson (1934) has a similar standard. Holm (quoted by Kayne, 1936) requires

at least an infiltration of 7 mm. diameter at forty-eight hours, or 5 mm. at seventy-two hours, for a positive reaction. Kayne (1936) adopts the following criteria in considering a reaction positive:

At 48 hours: area of erythema, with or without swelling, 10 mm. in diameter (mean).

At 72 hours: area of erythema and swelling, 7 mm. in diameter (mean).

TESTS BY G. GREGORY KAYNE

The erythema and swelling cannot always be measured with accuracy, and the "experimental error" of the size of the reaction has not been determined. It appeared, therefore, that as the object of the present investigation was to assist in clinical work, the comparisons should be based on the relative number of positive and negative reactions. The criteria adopted in considering a reaction positive with either substance are given above.

A total number of 119 persons were tested with old tuberculin and P.P.D. at comparable sites on each forearm: 114 were adult patients at the Clare Hall Sanatorium, Middlesex, all of whom except three suffered, or had recently suffered, from active pulmonary tuberculosis; the remaining five belonged to the domestic staff, and were healthy (three of them proved negative to all dilutions of both substances). All the results were read after twenty-four, forty-eight, and seventy-two hours, and the reactions were carefully measured. The subjects were injected first with the highest dilutions—namely, 1 in 10,000 of each preparation. If the tests were negative or doubtful they were repeated with the next stronger dilution. The results are as follows:

TABLE I.—Summary of Tests with Purified Protein Derivative and Old Tuberculin

	Dilutions		
	1 in 10,000	1 in 1,000*	1 in 100
P.P.D.—O.T.—	26 patients	7 patients	4 patients†
P.P.D.+O.T.+	63 "	24 "	2 "
P.P.D.+O.T.—	29 "	1 "	0 "
P.P.D.—O.T.+	1 "	0 "	0 "

* Some of the tests with 1 in 10,000 that were only just positive were repeated with 1 in 1,000.

† Three of these persons belonged to the domestic staff; the fourth was a tuberculous patient who died two days later.

TABLE II.—Variation of Readings (48 and 72 Hours)

Negative at 48 hours, positive at 72 hours ..	{ P.P.D. .. 3 patients O.T. .. 7 "
Positive at 48 hours, negative at 72 hours ..	{ P.P.D. .. 3 " O.T. .. 4 "

Note.—In each of these four groups were tests with both the 1 in 10,000 and the 1 in 1,000 dilutions.

An appreciably greater percentage of positive reactions is obtained with P.P.D. than with old tuberculin (Table I). A few reactions did not appear till seventy-two hours after the injections (Table II); and, with both old tuberculin and P.P.D., reactions occasionally occurred at twenty-four and forty-eight hours, which disappeared twenty-four hours later.

TESTS BY A. T. DOIG, G. GEMMILL, AND J. S. WESTWATER

In the present investigation we adopted the following minimum standard to be accepted as a positive reaction: an erythema of at least 10 mm. or an infiltration of at least 5 mm., in either case persisting for forty-eight hours. Reactions less than that were considered negative. We consider it would not be safe to accept a simple erythema under 10 mm. as a positive reaction without confirming the result by using a stronger dilution. It

should be noted that it is rare to have a 5 mm. infiltration without some surrounding erythema.

By taking the forty-eight-hour reading, any transient erythema arising from "needle trauma" is excluded, as this does not last longer than twenty-four hours. In this section of the work the forty-eight-hour reading, being usually the maximum, was taken as the standard for comparison of the two tuberculins, and the mean diameter was recorded in millimetres. In order to allow for errors of technique it was decided to regard as equal those reactions in which the mean diameter of the larger did not exceed that of the smaller by more than 25 per cent.*

A total of 302 subjects have been tested, the majority in the course of routine diagnostic work on children and adults. Of these, 241 can be considered adequately tested—that is, negative reactions were checked up to the 1 in 100 dilution—and 228 reacted to either or both forms of tuberculin.

In Table III the results are compared by measurement of the mean diameter. In 60.1 per cent. the reactions were equal; in 27.2 per cent. the reaction with P.P.D. was the

TABLE III.—Comparison of Purified Protein Derivative and Old Tuberculin by Measurement of the Mean Diameter of Reactions

Comparison of Mean Diameter	Dilution									Total	Per Cent.
	1/10,000			1/1,000			1/100				
	A.T.D.	G.G.	J.S.W.	A.T.D.	G.G.	J.S.W.	A.T.D.	G.G.	J.S.W.		
P.P.D.=O.T.	44	23	19	14	20	8	7	2	-	137	60.1
*P.P.D.>O.T.	25	15	3	10	6	-	2	1	-	62	27.2
†P.P.D.<O.T.	11	4	4	5	2	2	1	-	-	29	12.7
	Total ..									228	100

* Includes 24 cases in which reaction to P.P.D. was positive and to O.T. negative.

† Includes 5 cases in which reaction to P.P.D. was negative and to O.T. positive.

greater; while in 12.7 per cent. it was smaller than the response to old tuberculin. This would suggest that the dilutions of P.P.D. were slightly more potent, as they appeared to be in tests on guinea-pigs. The possibility of the greater stability of P.P.D. being a factor in eliciting a stronger reaction must be borne in mind, although the care taken to use the dilution of the old tuberculin within three weeks of preparation was considered to eliminate this

TABLE IV.—Comparison of Purified Protein Derivative and Old Tuberculin by Incidence of Positive and Negative Reactions

Reactions	A.T.D.	G.G.	J.S.W.	Total	Per Cent.
P.P.D.+ O.T.+ ..	97	64	35	196	86.0
P.P.D.+ O.T.— ..	19	7	—	26	11.4
P.P.D.— O.T.+ ..	3	2	1	6	2.6
	Total ..			228	100

factor, and Kayne's and Doig's observations (given below) confirm this view. In Table IV, comparing the tuberculins simply by the incidence of negative and positive

* Twenty-three subjects were injected by one of us (G. G.) in both arms at comparable sites with the same material taken from the same batch—namely, 0.1 c.cm. of P.P.D. diluted 1 in 1,000, or, for some tests, 1 in 10,000. The difference in the mean diameter of the individual reactions on both arms was usually 3 mm. or less, the extreme difference being 6 mm. in one comparison. The means of the mean diameters of all the reactions on right and left arms respectively were almost identical—namely, 22.4 and 22.6 mm. These results suggest that the margin of error—namely, 25 per cent. difference—was adequate, which was taken into consideration for the comparison of P.P.D. and old tuberculin.

reactions, the indication of greater potency of the dilutions of P.P.D. is repeated.

It is important to note that, despite the allowance of 25 per cent. in measuring the reactions to cover experimental error, there is considerable divergence in the results, showing that if the Mantoux technique is to be used as a means of standardization it is necessary that tests be made on considerable numbers of patients before any reasonable assessment of potency can be made. Madsen and Holm (1935) consider the intracutaneous test in humans a method of standardization preferable to tests in the sensitized guinea-pig. The necessity for a large series, however, has to be met. Multiple tests on the one subject have obvious objections, although Philip *et al.* (1930) considered it a practical method, using serial dilutions with the cutaneous (Pirquet) technique.

The reactions obtained from P.P.D. are similar in character to the response to old tuberculin. They could not be said to be more clear-cut and easier to interpret.

(b) Mantoux Tests : Additional Investigations : The Stability of Old Tuberculin

1. TESTS BY G. GREGORY KAYNE

On the basis of tests on guinea-pigs Parish and Okell (1929) and Okell (1930) found that dilutions (1 in 500 to 1 in 4,000) of old tuberculin in saline had lost about 40 per cent. of their potency after three months' storage at 4° C., at room temperature, or at 37° C. As a working rule they consider that sterile dilutions of old tuberculin in saline may safely be used even after being kept for one month at room temperature. Douglas and Hartley (1934) detected no loss of activity when dilutions of international standard old tuberculin in 0.5 per cent. phenol saline were stored at 2° C. and at room temperature for fifty-three days. Deterioration was observed, however, when they were stored at 37° C. for fifty-three days.

The opinion is commonly expressed, nevertheless, that dilutions of old tuberculin should not be used when more than two weeks old. Mantoux tests in human subjects were therefore carried out to investigate this statement, and a test made of the stability of dilutions of international standard old tuberculin in 0.5 per cent. phenol saline kept at various temperatures.

The different dilutions were used "blindly"—that is, they were labelled with symbols at the Wellcome Laboratories and the key was supplied to me later. This procedure is necessary so as to avoid the influence of preconceived ideas in reading reactions that are not sharply outlined. Since as many as four injections were made on one arm, the order in which dilutions were injected in each patient was varied to obviate the possible influence of the texture of the skin. Readings were taken at forty-eight and seventy-two hours, and for these investigations a dilution of 1 in 10,000 was used.

The results are shown in Tables V and VI.

TABLE V.—Three Dilutions of Old Tuberculin kept in the Ice-chest 9 Weeks, 7 Weeks, and 5 Weeks Respectively, and a Dilution Prepared the Previous Day

Readings	Results (Number of Positives) in 20 Patients			
	Fresh	5 weeks old	7 weeks old	9 weeks old
At 48 hours	18	18	17	17
At 72 hours	16	17	16	17
(19 patients only read)				

Allowing for some experimental error in reading, there appears to be no deterioration of the tuberculin even after nine weeks at 4° C.

TABLE VI.—*Four Dilutions of Old Tuberculin kept at Room Temperature (22° C.) for 11 Weeks, at 37° C. for 11 Weeks, at Room Temperature for 7 Weeks, and at 37° C. for 7 Weeks Respectively*

Results (Number of Positives)					
Patients Read	At 48 Hours		Patients Read	At 72 Hours	
	Fresh	11 weeks 22° C.		Fresh	11 weeks 22° C.
20	14 (70%)	10 (50%)	20	16 (80%)	9 (45%)
26	Fresh 18 (70%)	11 weeks 37° C. 9 (35%)	26	Fresh 17 (65%)	11 weeks 37° C. 11 (42%)
23	Fresh 17 (74%)	7 weeks 22° C. 14 (61%)	22	Fresh 12 (54%)	7 weeks 22° C. 12 (54%)
22	Fresh 16 (73%)	7 weeks 37° C. 12 (54%)	21	Fresh 15 (71%)	7 weeks 37° C. 12 (57%)

Since I consider the reading at seventy-two hours should be the one adopted for the Mantoux test, it appears that there is no deterioration of the tuberculin kept for seven weeks at room temperature, but definite deterioration occurs if kept for this period at 37° C. After eleven weeks there is definite deterioration if the tuberculin is kept at either of these temperatures.

In conclusion, the common opinion that dilutions kept for two weeks should not be used errs very much on the side of caution. Dilutions may be employed with safety up to six weeks, at all events if kept at room temperature, and for nine weeks if kept in an ice-chest.

2. TESTS BY A. T. DOIG

On the lines described above a test was made of the stability of 1 in 10,000 dilutions of old tuberculin kept at 4° C. for four weeks and for eleven weeks. The injections were made about the middle of the forearm (one on the right arm and two on the left), and in the same order on each patient. The skin in the elbow and wrist regions was avoided, but otherwise possible differences in the texture of the skin were disregarded.

Twenty-six patients were tested, of whom four did not react to any of the dilutions. The readings, taken at forty-eight hours, were:

1 in 10,000 dilution O.T.	3 days old ..	21 positive (1 doubtful)
" " "	" 4 weeks old ..	22 "
" " "	" 11 " " ..	21 "

It is therefore concluded that old tuberculin, even in dilutions of 1 in 10,000, is a considerably more stable substance than is commonly believed, and storage for as long as eleven weeks at 4° C. causes little appreciable loss of potency.

Experiment to Test the Accuracy of the Mantoux Test with Old Tuberculin

BY G. GREGORY KAYNE

When one of the dilutions investigated was 1 in 10,000 of international standard old tuberculin a 40 per cent. difference in potency between two dilutions was barely detectable, although a 60 per cent. difference had an appreciable influence on the number of positive reactions obtained (Table VII).

The differences in potency shown in the table suggest that we should examine once again the results of the Mantoux tests with P.P.D. and old tuberculin presented earlier in this paper. It will be remembered that the dilutions of the two preparations supplied from the Wellcome Laboratories were about equal in potency on intracutaneous tests in guinea-pigs; differences in the size of

the reactions were trifling, P.P.D. appearing to be the stronger only on the most sensitive animals.

TABLE VII.—*Four Dilutions of Old Tuberculin, 1/10,000, 1/12,500, 1/16,600, and 1/25,000, Corresponding to 100 per Cent., 80 per Cent., 60 per Cent., and 40 per Cent. of a Dilution of 1 in 10,000.*

Results (Number of Positives) in 24 patients				
	100%	80%	60%	40%
At 48 hours	16	18	17	13
At 72 hours	19	19	17	12

It is perhaps worthy of comment that few tuberculous guinea-pigs are sensitive to 1 in 10,000 dilutions of tuberculin, whereas many patients in sanatoria give large positive reactions to this dilution.

We are indebted to Dr. J. W. Trevan for the following statistical note:

"The experiments on guinea-pigs recorded earlier in this paper show a distribution which would, I calculate, occur by accident about eight times in a hundred trials on groups of forty-six guinea-pigs. Any difference between P.P.D. and O.T. for the guinea-pig is therefore not large enough to be established by the use of forty-six guinea-pigs with the degree of probability that is usually taken as significant, but the results are suggestive.

"The experiments recorded in Table III (228 patients), whether the total or only the 1 in 10,000 dilution figures are used, give a highly significant excess of P.P.D. > O.T. over P.P.D. < O.T. It can be taken as established that P.P.D. in the doses used is more potent than O.T. This is borne out by Table I (119 patients) and Table IV.

"But it cannot be established that P.P.D. is more potent than O.T. for man when given in doses which are identical on guinea-pigs, for there are more observations on man. It must not be assumed that since the differences in man are highly significant, whereas those on the guinea-pig are not significant by the ordinary criterion, there is a real difference between the relative potencies on man and on the guinea-pig. Further multiplication of tests on the guinea-pig might quite well result in the establishment of a significant difference between the dilution doses of P.P.D. and O.T. used for that animal."

Summary and Conclusions

1. Attention is directed to purified protein derivative (P.P.D.) as the most suitable reagent for the Mantoux test. It is the active principle isolated in a pure state as a dry powder from a synthetic medium old tuberculin. It corresponds to Seibert's SOTT (synthetic medium old tuberculin trichloroacetic-acid-precipitated). It is almost invariably of constant potency on a weight basis, is very stable in the dry state, and is of a non-sensitizing nature. It is therefore particularly suitable for use in large-scale surveys where uniformity in the amount of the active principle injected is essential.

2. "Dried dilutions" which are likewise of uniform potency and quite stable can also be prepared from P.P.D. As a precautionary measure the potency of these "dried dilutions" is checked from time to time against the international standard old tuberculin, the intracutaneous test in the tuberculous guinea-pig being used for the comparisons. On no occasion has any variation been observed from the calculated potency. The "dried dilutions" may be redissolved easily and quickly in a suitable borate buffer solution immediately before use.

3. A series of "dried dilutions" of P.P.D. have been compared with corresponding dilutions of international

standard old tuberculin by means of the Mantoux test, the two tuberculins having been titrated previously on sensitive guinea-pigs. On intracutaneous test in a large number of guinea-pigs the preparations were almost equal in potency, only the most sensitive animals indicating that the "dried dilutions" were a trifle stronger.

4. The reactions obtained with P.P.D. were very similar in character to the response with old tuberculin, both in guinea-pigs and in human subjects.

5. Clinical comparison, both by measurement and by the relative number of positive and negative reactions obtained, indicated that the P.P.D. was slightly more potent than the old tuberculin, a fact that was only evident on the most sensitive guinea-pigs.

6. The results of the four clinical workers suggest that if the Mantoux test were to be used as a method of comparing the potency of two tuberculins it would be necessary to have observations on a large number of patients before any conclusion could be drawn. There would appear to be no advantage in using human subjects instead of guinea-pigs for the titration of old tuberculin.

7. In practice the deterioration of dilutions of old tuberculin in phenol saline is not sufficient to prevent their use for Mantoux tests if they have been kept up to six weeks at room temperature or nine weeks in the ice-chest. "Dried dilutions" of P.P.D. are much more stable, and have shown no detectable loss in potency after three years at room temperature.

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W. Knoll (*Med. Klinik*, April 8, 1938, p. 464) has followed up the life history of 880 Oxford and Cambridge "Blues" and refutes the theory that as a rule they die before the age of 50 as a result of the severe strain on the heart during the training period. Of the 880 investigated, 27.5 per cent. took part in one inter-university boat race, 29.5 per cent. in two, 24.5 per cent. in three, 14.5 per cent. in four, and 4 per cent. in more than four races. A statistical survey shows that by 1929 five-eighths of all the living participants in the races were above the age of 50, the average age being 56.1 years, and many having reached the age of 70 and over. Of 155 deaths, eight were due to infective diseases, seventeen to tropical diseases, twelve to accidents, and forty-five died in the war; thus the mortality from natural causes was 8.1 per cent., which figure is relatively low.

THE CAUSES OF HAEMORRHAGE FROM THE RECTUM*

BY

J. P. LOCKHART-MUMMERY, F.R.C.S.

Emeritus Surgeon to St. Mark's Hospital for Diseases of the Bowel

Visible blood may be passed per anum under a variety of circumstances and from many different causes. Such an occurrence is always alarming to the person concerned, and the medical attendant will at once be called upon to give a reason for it and reassure the patient as to its meaning. It will be necessary to account for the bleeding at once, or at least to arrange for some examination to be made which will reveal the cause. While it is advisable to reassure the patient, it is never wise to tell him or her that a little bleeding is of no serious consequence and to treat it by ordering a few suppositories. Such guesswork methods are dangerous both to the patient's well-being and to the doctor's reputation. It is therefore of some importance to consider what the cause of the haemorrhage may be and how the source and reason for the bleeding may best be ascertained.

Naturally a large haemorrhage will give rise to much more alarm than a small one, but it does not follow that the former condition is the more serious or has the gravest significance. Any bleeding, whether small in quantity or not, calls for immediate attention, and should never be neglected, as not infrequently very slight intermittent bleeding is the first sign of a malignant growth somewhere in the intestinal tract; nor is it safe to assume that because the patient is quite a young person malignant disease can be ruled out. I have seen a child of 8 years with carcinoma of the rectum, and although the condition is rare under the age of 40 there are quite enough cases to make us very cautious.

It is a curious and not unimportant fact that patients who have suffered for some months from what they describe as "slight bleeding" at stool often exhibit a much more serious degree of anaemia than those who have had one large haemorrhage. The probable reason for this is that patients are prone to attach much less importance to small often-repeated bleedings than to a single severe one.

Internal Piles

The commonest cause of haemorrhage from the rectum is unquestionably internal piles, and this probably accounts for quite two-thirds of all the cases of bleeding from the rectum. I have seen patients who, as the result of frequent haemorrhages due to piles, have been bled white and whose blood count had been reduced to under 3,000,000 red cells per c.mm. Not long ago I had in this hospital a patient with internal piles who was a professional pugilist. He had been bleeding at stool for over a year, and he was so reduced by it that he could hardly walk across a room without feeling faint. His skin was a greenish white and his red cell count was under 3,000,000 per c.mm. Before operating for the piles it was necessary to transfuse him with human blood. But such severe anaemia is unusual as the result of piles.

Bleeding due to piles can generally be recognized, because a simple examination of the rectum with a speculum will reveal the presence of easily prolapsible internal piles, and also because the characteristic form of bleeding is for the blood to drip or squirt after the bowel

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