THE STANDARDISATION OF TUBERCULIN.

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(With Plates VII–XII.)

Tuberculin is a term which has been employed loosely by successive investigators to describe preparations obtained by various methods from cultures of *Bacillus tuberculosis* reared on media of varying constitution. It is perhaps unfortunate that from the first the use of the term had not been limited to products whose preparation conformed to that of Koch's original tuberculin.

The original tuberculin (Old Tuberculin) is a glycerinated, concentrated filtrate from a culture of the bacillus on nutrient broth not less than 6 weeks old, concentration being effected by evaporation to one-tenth of the original volume of the fluid. Had the subsequent production of tuberculins followed Koch's method strictly, the potency of the resulting products would have been approximately uniform. The course of events has been otherwise. Formulae of media, and methods of production, have varied much. Even products professedly obtained according to Koch's methods have been found to vary in potency. This has led to inaccuracy of thought and statement.

Koch recognised the need for standardisation and proposed to base it on the lethal effect of a given quantity of tuberculin on a tuberculous guinea-pig: 0·5 c.c. of the liquid product should kill a tuberculised guinea-pig. The procedure has the merit of directness and simplicity, but has obvious limitations. On either side of the lethal line there is possibility of wide variation in potency. The procedure overlooks variation in reactivity of tuberculised guinea-pigs. The method is of little use for assessment of therapeutic and diagnostic values.

Other methods of standardisation have been proposed from time to time. None of these can be accepted without qualification.

Were the essential elements of tuberculin certainly determined, a chemical standard might be attained. In the absence of such determination, this is excluded. Further investigations into the constitution of tuberculin may guide in that direction. Meanwhile, it is to be borne in mind that even the chemical composition of culture media for the growth of the tubercle bacillus is by no means fixed.

Short of chemical tests, we might fall back on biological facts and simply define tuberculin as "that substance which elicits what by common consent is called the tuberculin reaction," that is, the allergic reaction yielded by the tuberculised animal, *e.g.* to the intracutaneous test. In this way tuberculin

might be standardised by reference to the degree of reaction in the sensitised animal.

Unfortunately, there is wide variation in reaction among sensitised animals. This is a disturbing admission in assessing the value of the procedure. It would be necessary in standardisation of a new preparation to compare the results of its application with those obtained by the use of an accepted standard on the same animal.

A further application of the principle has been devised by Long (1926), based on the hyper-sensitiveness of the germinal cells of the tuberculous animal —what he terms the spermatocyte reaction—which is stated to be specific for tuberculin. The method, which rests on the degree of change determined microscopically in the testicle of the tuberculised guinea-pig which has been injected with tuberculin, cannot yet be included within the practicable ambit.

Various serological methods have been proposed. Had their validity been established, they would offer the advantage of a unit of measurement for the dosage of tuberculin. Such methods include an application of complement fixation procedure (Watson and Heath, 1924). Evidence is lacking, however, as to whether the results obtained are essentially related to the tuberculin reaction. It is maintained that culture media in use are themselves possessed of antigenic properties of very varying degree and that a purified tuberculin, free from extraneous products, fails as regards complement fixation (Calmette, 1922).

The precipitin method of Dreyer and Vollum (1924) is subject to similar criticism. It seems open to question whether this and the tuberculin reaction are correlated. It appears that some potent tuberculins give a weak response to the precipitin test, and that highly purified tuberculins may give a weaker result than crude preparations.

The question of standardisation of tuberculin has been studied by the Health Committee of the League of Nations during the past three or four years. In April 1926 a *Report* was issued by the League over the names of Prof. Calmette and Dr de Potter. Their conclusions were to the effect that serological methods which had been proposed were of little value, that Koch's method was only of partial value, and that the nearest approach to standardisation was obtainable by injection of the various tuberculins into the dermis of a tuberculised animal, and assessment of their comparative potency by reference to results obtained by injection of a selected tuberculin as standard.

In view of results to be recorded in the present investigation, it is especially noteworthy that the conclusions of the Health Committee's *Report* favour standardisation tests by intracutaneous injection as against cutaneous (Pirquet) applications, on the ground that the results afforded by the latter are less precise, and that the cutaneous application is more disturbing to the human subject under observation than is the intracutaneous injection. The results to be submitted in the subsequent pages, which are based on extensive observations, point towards different conclusions.

So far as Great Britain is concerned, the need for a practical method of standardisation has become imperative by reason of the passage of the Therapeutic Substances Act, 1925, and the issue of the Therapeutic Substances Regulations, 1927, by the Joint Committee constituted under sub-section (1) of Section 4 of the Act.

The Second Schedule appended to the Regulations, in so far as it applies to the subject under consideration, reads as follows:

TUBERCULINS.

1. (1) Tuberculins are preparations of fluid media on which the *Bacillus tuberculosis* has been grown in artificial culture and which have been freed by filtration from the bacilli.

2. (1) Old Tuberculin is the concentrated filtrate from the growth of *Bacillus tuberculosis* on a suitable nutrient broth. For its preparation the bacillus must be grown at approximately 37° C. for a period, usually not less than 6 weeks, sufficient to allow the surface of the fluid medium to become covered by a thick growth of the bacillus. At the end of this period the fluid medium, from which the bacilli may or may not have been previously separated by filtration, must be concentrated by evaporation to one-tenth of its original volume, and then filtered. If the required test for potency shows that the preparation so concentrated is more potent than the standard preparation, the potency may be reduced to the required degree by appropriate dilution. If the test shows that the potency is less than that of the standard preparation, it shall not be increased by further evaporation. The proper name of the preparation is "Old Tuberculin," with or without a suffix such as T., or P.T. The suffix T., if used, must be used to indicate that the bacillus used in preparing the Tuberculin was obtained from a case of human infection; and the suffix P.T. to indicate that the bacillus used was obtained from a case of bovine infection.

(2) The standard preparation of Old Tuberculin is a quantity of Old Tuberculin kept in the National Institute for Medical Research, Hampstead.

(3) Each batch of Old Tuberculin shall be tested for potency by observation of its specific toxicity for guinea-pigs or other animals infected with the *Bacillus tuberculosis*. The test shall be made by a method approved by the Licensing Authority, and shall be such that the potency of the preparation under test is measured by comparison with that of the standard preparation. Old Tuberculin shall not be issued if its activity differs from that of the standard preparation to such an extent that the difference is revealed by the test.

(4) Each batch of Old Tuberculin shall be tested for the absence of non-specific toxicity by the subcutaneous injection of 0·5 c.c. into a normal guinea-pig, and shall be treated as having passed the test if such injection does not cause death or serious symptoms.

The immediate issue is thus limited to preparations professing to be of the nature and value of "Old Tuberculin." None the less, some of the results to be obtained have wider bearings.

It will be noted that standardisation is based arbitrarily on comparison of a given tuberculin with "the standard preparation of Old Tuberculin kept in the National Institute for Medical Research, Hampstead." Each sample is subject to two tests: (1) for potency, (2) for the absence of non-specific toxicity.

The latter test is readily effected by the subcutaneous injection of 0·5 c.c. into a normal guinea-pig. The result is satisfactory if the injection fails to kill or cause serious symptoms.

The former test, that is, the test for potency, "shall be made by a method

approved by the Licensing Authority, and shall be such that the potency of the preparation under test is measured by comparison with that of the standard preparation."

The object of the present research was to explore the applicability for test purposes of results obtained by means of the cutaneous reaction (Pirquet) in the human subject, and to assess their value by comparison with results obtained by means of the intra-cutaneous reaction in animals. Should the two sets of results be proved to be similar and approximately equivalent, and the test be found innocuous to the subject under observation, the former procedure had the obvious advantage that, based on facts determined directly in relation to human tuberculised subjects, the deductions were more immediately applicable for the various purposes of diagnosis and treatment in man, one of the chief purposes of standardisation being to ensure that the potency of a given preparation is up to standard.

Standardisation of tuberculin by means of the cutaneous test.

The cutaneous reaction as described by Pirquet was used as the basis of the following work. It was ascertained what degree of reaction occurred in tuberculous human subjects when various strengths of the same tuberculin were applied to the arm. A tuberculin was applied in the following strengths (diluted with sterile 0·5 % carbol-saline solution): 1/1, 1/2, 1/4, 1/8, 1/16, and 1/32; the Pirquet method being used for the scarifications. It was found over a considerable number of patients that the degree of reaction was directly proportional to the concentration of the tuberculin, the more concentrated the tuberculin the greater the degree of reaction. It was further found that the same tuberculin gave similar results in both arms of the same patient. And finally, after extensive trial, it was found that neither the glycerine contained in the pure tuberculin nor the carbol-saline with which it was diluted, produced a reaction on the skin surface when applied *per se*.

In the first series of observations an unknown tuberculin was tested against a tuberculin of ascertained strength. Each tuberculin was applied in the above dilutions to the flexor surface of the forearm of the selected patient—a known tuberculin reactor. Within 6 to 10 hours definite reactions could be recognised, more or less circular in outline, concentric round the scarified area. The next question was the best way of recording each reaction. It appeared that the most accurate way to arrive at the degree of intensity of reaction was to measure its diameter. Accordingly the reactions were measured in millimetres, in terms of the average diameter, at intervals of 12, 18, 24, 36, 48, 60, and 72 hours after application of the tuberculin. It was found in every one of the cases examined that the maximum reaction—that is, the reaction when most clearly outlined against the white background of the patient's skin—was attained after the lapse of 48 hours, and that thereafter it tended to decrease in intensity. Accordingly, in subsequent records of results, the measurements were made 48 hours after the application of tuberculin.

In all the patients, careful watch was kept for 48 hours after the application of the tuberculin for the incidence of any focal and general reactions. In no case did any difficulty arise. It was therefore concluded that no reasonable objection could be raised to using the human subject as the medium for further observations.

The early results were encouraging. In practically every instance the tested tuberculins gave reactions whose area was proportional to their concentrations as in the earlier trials. In the majority of the patients, dilutions 1/16 and 1/32 gave very little or no reaction. It was thus possible to compare one tuberculin with another, both being applied under the same conditions.

At this stage the objection presented itself that the scarified areas were not quite uniform in size and shape, and that, therefore, there might be a considerable degree of experimental error in the results. It was felt also that if the method was to be elaborated further, that is, if numerous scarifications were to be made on the same patient, some method involving less time and less strain on the patient, and likewise ensuring uniformity of procedure should be found.

After extended experiment the following technique was evolved. A burr, after the fashion of that employed by a dentist, having a serrated circular end, 3 mm. in diameter, was adopted for the scarifications. With this instrument it was found possible, after a little practice, to produce a uniform circular scarified area (Plate VII). After each scarification, one drop of the tuberculin under examination was applied and allowed to dry on the skin. Each scarification could be made in a fraction of a second, and twenty-four such areas, which the later series entailed, along with the application of the tuberculin dilutions, could be completed within 1 or 2 minutes. As before, readings were taken of the diameters at the end of 48 hours.

A definite technique having been attained, a further series of observations was undertaken. A number of tuberculins, of unknown strength, were diluted as before and compared with the selected standard. It was found that three tuberculins and the standard could with ease and safety be compared in all the dilutions on the same patient. As before, the results were satisfactory, and readings were made without difficulty 48 hours after the original application.

As a result of these extended observations, it was found that there was a constant relationship between the area of reaction and the concentration of tuberculin applied. In estimating the strength of various samples this relationship has proved of the first importance.

Plates VIII and IX[1] show the appearances presented by the two arms in the same subject when the reaction is negative.

Plate X shows approximately equal reactions in the two arms—the same tuberculin being used for both arms—namely, a positive reaction in decreasing

[1] The Plates are from photographs, taken 48 hours after the several applications, the outline of the areas involved in some instances being reinforced by painting the skin surface lightly with ordinary red ink.

degree of intensity following the application of 100, 50, 25, and 12·5 per cent. respectively, a faint increase of reaction following 6·25 per cent., and a negative reaction to the weakest dilution (3·125 per cent.), and likewise to the control application.

REPORT ON TESTS CARRIED OUT ON HUMAN SUBJECTS.

1. The following tuberculins (*B*, *C*, *D*, *E* and *F*) were compared with the Standard tuberculin (human), issued by the Medical Research Council, London (*A*):

B. Tuberculin (T.), R.C.P. Edinburgh, Batch 4, June 1928.

C., *D*., *E*., *F*. Various tuberculins received severally from recognised producers.

2. Each tuberculin was tested in the following strengths: 1/1, 1/2, 1/4, 1/8, 1/16, 1/32 (diluted with carbol-saline immediately previous to application).

3. Two series of observations were carried out, and in each five patients were employed.

In Series I, tuberculins *A*, *B*, *C* and *D* were tested together on the same patient, *i.e.* tuberculins *B*, *C* and *D* were compared with the standard *A*.

In Series II, *A*, *B*, *E* and *F* were tested together, *i.e.* tuberculins *B*, *E* and *F* were compared with the standard *A*.

4. *Technique*. The flexor surface of the forearm was cleansed with methylated ether. Scarification of the skin, prior to the application of tuberculin, was carried out in all cases with the special burr (see Pl. VII, figs. 1 and 2), so as to ensure that each scarification was of similar size and approximately equal intensity.

Thereafter each dilution of tuberculin was applied to the appropriate area. No means were employed to rub the tuberculin in over the scarified area, one drop merely being placed on the area and allowed to dry.

The order of application (see photographs) is as follows, reading from right to left:

	Right arm		Left arm	
Series I	*A*	*B*	*C*	*D*
Series II	*A*	*B*	*E*	*F*

The dilutions of tuberculin were applied in the following order, reading proximo-distally: 1/1, 1/2, 1/4, 1/8, 1/16, 1/32.

5. *Results*. The intensity of the reaction was recorded in terms of the diameter of reaction. The medio-lateral and proximo-distal diameters were measured in millimetres and the average taken as the diameter of reaction.

Readings were taken after 12, 24, 36 and 48 hours. As in every case the reaction was most intense and most clearly defined after 48 hours, the readings taken then are appended.

Illustrations of the results obtained will be found on Plates XI and XII.

Series I. *Samples* B, C *and* D *compared with the standard* A.

Date of test	9. vii. 28	11 a.m.
Date of reading results	11. vii. 28	11 a.m.
Date of photographs...	11. vii. 28	11.30 a.m.

N.B. The figures indicate the diameter of reaction in mm.

1. S. D.

Right arm		Left arm	
A	*B*	*C*	*D*
16	16	14	15·5
15	16	12·5	15
14	12	12	15
12	10	9	12·5
11·5	10	7	12
7	7	Negative	9

2. J. D.

Right arm		Left arm	
A	*B*	*C*	*D*
16	17	15	15·5
14	14	12·5	13
12	12	7	7
11·5	9	5	6
9	6	Negative	4·5
Negative	5	,,	Negative

3. W. H.

Right arm		Left arm	
A	*B*	*C*	*D*
13·5	20	19	18
15	19	12	15
14	14	10	14
11	12	8	14
8	9	6	12·5
6	7	Negative	10

4. W. H.

Right arm		Left arm	
A	*B*	*C*	*D*
12	14	9·5	15
9	10	7	9
7·5	8	Negative	6·5
5	6·5	,,	Negative
Negative	Negative	,,	,,
,,	,,	,,	,,

5. J. P.

Right arm		Left arm	
A	*B*	*C*	*D*
14	15	11·5	17
12·5	14	11	15·5
13	14	10·5	13
12	12·5	7	12·5
9	8·5	Negative	9·5
7·5	8	,,	6·5

Series II. *Samples* B, E *and* F *compared with the standard* A.

Date of test	16. vii. 28	10 a.m.
Date of reading result	18. vii. 28	10 a.m.
Date of photographs	18. vii. 28	11 a.m.

N.B. The figures indicate the diameter of reaction in mm.

6. R. N.

Right arm		Left arm	
A	*B*	*E*	*F*
17	18·5	15	8
17	18	15	6
16	16	13	Negative
12	13	9·5	,,
10·5	11·5	Negative	,,
7	6	,,	,,

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7. K. W.	Right arm		Left arm	
	A	*B*	*E*	*F*
	23·5	22·5	20	10
	18	18·5	19	8
	17	16·5	17	Negative
	15·5	15	11	,,
	14	12·5	8	,,
	10	9·5	Negative	,,

8. J. M.	Right arm		Left arm	
	A	*B*	*E*	*F*
	19·5	20	15	12·5
	17	18	18	10
	16·5	16·5	14·5	6·5
	14	16	10	Negative
	9·5	8	7·5	,,
	5	5	Negative	,,

9. C. C.	Right arm		Left arm	
	A	*B*	*E*	*F*
	20	24	17	5
	16	17·5	12·5	4
	13·5	14	8	Negative
	10	11	6·5	,,
	7	7	Negative	,,
	5·5	5·5	,,	,,

10. A. D.	Right arm		Left arm	
	A	*B*	*E*	*F*
	15·5	15·5	15	8
	15	20	15	7
	14	20	14	Negative
	13·5	14	11·5	,,
	10	9	Negative	,,
	Negative	Negative	,,	,,

With a view to ready assessment of results the observations may be conveniently tabulated as shown in Tables I–V.

Table I. *Standard* (A) *and tuberculin* B.

	1/1		1/2		1/4		1/8		1/16		1/32	
No.	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*
Series I												
1	16	16	15	16	14	12	12	10	11·5	10	7	7
2	16	17	14	14	12	12	11·5	9	9	6	—	5
3	13·5	20	15	19	14	14	11	12	8	9	6	7
4	12	12	9	10	7·5	8	5	6·5	—	—	—	—
5	14	15	12·5	14	13	14	12	11·5	9	8·5	7·5	8
Series II												
6	17	18·5	17	18	16	16	12	13	10·5	11·5	7	6
7	23·5	22·5	18	18·5	17	16·5	15·5	15	14	12·5	10	9·5
8	19·5	20	17	18	16·5	16·5	14	16	9·5	8	5	5
9	20	24	16	17·5	13·5	14	10	11	7	7	5·5	5·5
10	15·5	15·5	15	20	14	20	13·5	14	10	9	—	—

Table II. *Standard* (A) *and tuberculin* C.

	1/1		1/2		1/4		1/8		1/16		1/32	
No.	*A*	*C*	*A*	*C*	*A*	*C*	*A*	*C*	*A*	*C*	*A*	*C*
1	16	14	15	12·5	14	12	12	9	11·5	7	7	—
2	16	15	14	12·5	12	7	11·5	5	9	—	—	—
3	13·5	17	15	12	14	10	11	8	8	6	6	—
4	12	9·5	9	7	7·5	—	5	—	—	—	—	—
5	14	11·5	12·5	11	13	10·5	12	7	9	—	7·5	—

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Table III. *Standard* (A) *and tuberculin* D.

	1/1		1/2		1/4		1/8		1/16		1/32	
No.	*A*	*D*	*A*	*D*	*A*	*D*	*A*	*D*	*A*	*D*	*A*	*D*
1	16	15·5	15	15	14	15	12	12·5	11·5	12	7	9
2	16	15·5	14	13	12	7	11·5	6	9	4·5	—	—
3	13·5	18	15	15	14	14	11	14	8	12·5	6	10
4	12	15	9	9	7·5	6·5	5	—	—	—	—	—
5	14	17	12·5	15·5	13	13	12	12·5	9	9·5	7·5	6·5

Table IV. *Standard* (A) *and tuberculin* E.

	1/1		1/2		1/4		1/8		1/16		1/32	
No.	*A*	*E*	*A*	*E*	*A*	*E*	*A*	*E*	*A*	*E*	*A*	*E*
1	17	15	17	15	16	13	12	9·5	10·5	8	7	—
2	23·5	20	18	19	17	17	15·5	11	14	8	10	—
3	19·5	15	19	18	16·5	14·5	14	10	9·5	7·5	5	—
4	20	17	16	12·5	13·5	8	10	6·5	7	—	5·8	—
5	15·5	15	15	15	14	14	13·5	11·8	10	—	—	—

Table V. *Standard* (A) *and tuberculin* F.

	1/1		1/2		1/4		1/8		1/16		1/32	
No.	*A*	*F*	*A*	*F*	*A*	*F*	*A*	*F*	*A*	*F*	*A*	*F*
6	17	8	17	6	16	—	12	—	10·5	—	7	—
7	23·5	10	18	8	17	—	15·5	—	14	—	10	—
8	19·5	12·5	17	10	16·5	6·5	14	—	9·5	—	5	—
9	20	5	16	4	13·5	—	10	—	7	—	5·5	—
10	15·5	8	15	7	14	—	13·5	—	10	—	—	—

From the above tables, it will be observed that of the five tuberculins (*B*, *C*, *D*, *E*, and *F*), compared with the standard solution (A), the first four approximated to the standard in more or less uniform degree, while the remaining tuberculin *F* fell much below the standard. Tuberculin *F* was accordingly dropped from the further tests.

REPORT ON TESTS CARRIED OUT ON GUINEA-PIGS.

On 29th May 1928 guinea-pigs were inoculated subcutaneously in the groin each with an equal dose of living tubercle bacilli (human type) from a 14 days' culture on glycerin-egg medium.

In the sixth week following infection all showed an ulcerated lesion at the site of inoculation and enlargement of the neighbouring lymphatic glands. They were tested for hypersensitiveness with 0·1 c.c. of a 1/500 dilution of the Medical Research Council standard tuberculin (A) injected intradermally and all gave a positive reaction of varying intensity.

The following comparative tests with a number of selected tuberculins were then carried out[1]:

Five guinea-pigs were used for each test; they were prepared one or two days before use by removing the hair from the abdomen with scissors and depilating powder. On the day of the test dilutions in carbol-saline of 1/4000, 1/2000, 1/1000 and 1/500 of the tuberculin to be tested and of the Medical Research Council standard tuberculin (A) were prepared.

Each guinea-pig received 0·1 c.c. intradermally of the various dilutions of

[1] The following method was kindly communicated to Prof. Mackie, Edinburgh University, by Dr O'Brien of the Wellcome Physiological Research Laboratories.

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the tuberculin to be tested and of the standard, about three centimetres apart, and the average diameter of the area of reaction in millimetres was measured 24 hours later. These readings were checked by a second observer, and are given in the following tables.

Tuberculins A *and* B_1 (α) (8. vii. 28).

Guinea-pig No.	1/4000		1/2000		1/1000		1/500	
	A	B_1	*A*	B_1	*A*	B_1	*A*	B_1
1	8	7	10	8	12	11·5	16	14
2	4	4	6	5	8	8	12	12·5
3	6	6	8	6	9	9	16	14
4	8	7	10	10	12	11	14	13·5
5	4	4	5	5	9	7	12	8

Tuberculins A *and* B_1 (β) (24. vii. 28).

Guinea-pig No.	1/4000		1/2000		1/1000		1/500	
	A	B_1	*A*	B_1	*A*	B_1	*A*	B_1
1	5	5	5	10	14	14	18	20
2	—	—	—	—	—	—	—	—
3	7	3	7	6	10	11	17	15
4	5	3	5	5	10	7	16	14
5	4	4	7	9	13	12	16	14

Tuberculins A *and* B_2 (α) (20. vii. 28).

Guinea-pig No.	1/4000		1/2000		1/1000		1/500	
	A	B_2	*A*	B_2	*A*	B_2	*A*	B_2
1	4	4	8	5	10	6·5	11	11
2	4	2	5	6	6·5	7	14	9
3	5	6	8	12	10	10	22	16
4	5	4	5	5	10	10	15	15
5	5	5	8	6	12	10	16·5	17

Tuberculins A *and* B_2 (β) (31. vii. 28).

Guinea-pig No.	1/4000		1/2000		1/1000		1/500	
	A	B_2	*A*	B_2	*A*	B_2	*A*	B_2
1	4	4	5	5	11	9	15	16
2	5	4	7	10	10	11	14	16
3	3	3	5	5	11	10	15	16
4	3	3	6	5	8	8	12	12
5	3	3	4	5	8	7	14	11

Tuberculins A *and* B_3.

Guinea-pig No.	1/4000		1/2000		1/1000		1/500	
	A	B_3	*A*	B_3	*A*	B_3	*A*	B_3
1	5	4	7	7	12·5	11	16	13
2	4	5	7	8·5	11	14	17	14
3	5	4	8	7	10	11	15	15
4	3	3	4	4	10	8	13	11
5	8	8	12	10	15	12	17	16

Tuberculins A *and* C.

Guinea-pig No.	1/4000		1/2000		1/1000		1/500	
	A	*C*	*A*	*C*	*A*	*C*	*A*	*C*
1	7	7	8·5	11	11	13	17	19·5
2	7	9·5	11	12·5	13	16	19	20
3	7	10	8·5	11	10·5	9*	14·5	17
4	2	2	2	3	3	5	4	17
5	4	7	6	10	13	12	19	18

* Owing to abrasions produced by the guinea-pig scratching itself, this reaction could be estimated only approximately.

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Tuberculins A *and* D.

Guinea-pig No.	1/4000		1/2000		1/1000		1/500	
	A	*D*	*A*	*D*	*A*	*D*	*A*	*D*
1	Nil	Nil	7	3	7	6	11	9
2	7	6	10	7	12·5	11·6	16·5	17·5
3	3	3	4	4	4·5	6	11·5	12
4	7	7	7	8	11	11	15	16·5
5	9	8	11	10·5	14	11	15·5	16

Tuberculins A *and* E.

Guinea-pig No.	1/4000		1/2000		1/1000		1/500	
	A	*E*	*A*	*E*	*A*	*E*	*A*	*E*
1	7	7	9	8	12	10	16	15
2	4	7	4	9	8	12	12·5	14
3	6	5	8	5	10	8	17	12
4	5	7	9	8	12	12	17	17
5	4	5	6	6	7·5	7	12·5	11·5

Tuberculin A, 100 *per cent. and* 50 *per cent.*

Guinea-pig No.	1/4000		1/2000		1/1000		1/500	
	100 %	50 %	100 %	50 %	100 %	50 %	100 %	50 %
1	10	10	12	11	15	13	18	14
2	6	3	8	5	12	11	15	12
3	8	8	11	10	15	12	18	13
4	9	4	13	9	16	12	19	15
5	4	2	7	4	10	8	16	11

Tuberculin A, 100 *per cent. and* 75 *per cent.*

Guinea-pig No.	1/4000		1/2000		1/1000		1/500	
	100 %	75 %	100 %	75 %	100 %	75 %	100 %	75 %
1	8	7	13	12	17	13	20	17
2	7	7	12	12	15	14	18	15
3	3	2	5	5	10	8	15	11
4	6	5	11	11	15	13	18	16
5	5	5	10	7	14	11	18	16

ANALYSIS OF DATA.

In order to compare the relative strengths of two tuberculins, a group of five suitable humans or guinea-pigs was treated under standard conditions as described in the experimental section, each with a series of dilutions of a standard which we shall call tuberculin *A*, and of tuberculin *B*, the strength of which it was desired to determine. After a given time, so chosen that the local reactions were approximately at their maximum, the diameters of the approximately circular inflamed regions were measured. It was observed in a series of inoculations on the same subject with different concentrations of one tuberculin, that this diameter increased with the concentration of the tuberculin, and it was apparent that if the law were ascertained which gave the relation between the diameter and the strength of the sample, a method could be devised for comparing the strengths of the two samples capable of giving a fairly accurate result with a small number of animals. It was necessary then, first of all, to discover approximately the relation between the diameter of the inflamed region and the strength of the sample. It should be made clear at this

stage that any law so discovered could be true only in a statistical sense, since considerable variations occur, owing presumably to experimental errors and local variations from point to point in the skin of the same subject. It will, however, appear in the sequel that on the average the area of the inflamed region is proportional to the strength of the tuberculin applied. In the case of any particular tuberculin the ratio of this area to the concentration which has been applied will be high in the case of a strong tuberculin, and low if the tuberculin is weak, for a subject of given sensitivity. The subjects, however, vary in their sensitivity but from each subject a single index of the relative strengths can be obtained, since, as mentioned above, a series of observations with the two tuberculins is carried out on the same subject. As the individual observations are subject to considerable error this index will also be subject to variation. In practice five subjects have been used to compare each unknown tuberculin in terms of the standard. In the case of the guinea-pigs four dilutions were applied to each animal; so that the final figure for each tuberculin was based upon 40 independent observations; in the case of the human experiments six dilutions were employed, and in this case the number of observations was 60. Five indices are thus obtained, one from each subject, and it is now necessary to find from these five values the best measure of the strength of the unknown in terms of the standard, and also the probable error of the estimation. Convenient formulae have been deduced and applied to the experimental data. The method has also been applied to estimate known dilutions of the standard in terms of the standard and as shown below satisfactory results have been obtained.

The following discussion of the problem from the statistical point of view may be omitted by the non-mathematical reader.

In order to be able to compare the quantity of tuberculin which gives rise to an area of reaction of radius r_1 with that which gives rise to an area of radius r_2, it is necessary to know the relation between r_1 and the quantity of tuberculin q_1 which has been administered. Since in each animal a series of doses of the same tuberculin has been applied, it is possible to find this relationship from the experimental data. It is necessary to realise that the individual observations are each subject to various errors of technique and subjective judgment, and further that each animal possesses its own particular degree of sensitivity. It is therefore convenient to assume a formula for the relationship between the dose q and the radius r, which contains a constant depending on the sensitiveness of the particular animal. The formula

$$q = k_m r^s$$

suggests itself, where k_m refers to the sensitiveness of the mth animal. We have then to find the value of s which gives the best agreement with the observed results, and also the particular values of k_m referring to the individual animals.

A certain amount of arbitrariness arises, since various criteria may be

taken in order to determine the degree of agreement between the experimental observations and the assumed law. For instance, taking the above formula we might apply it in the form $q = k_m r^s$, or in the form $r = \left(\frac{q}{k_m}\right)^{1/s}$, and if the constants k_m and $1/s$ were determined by means of the law of least squares from these two forms of the above formulae, the results would be slightly different, although in all probability in comparatively close agreement. In practice considerations of arithmetical simplicity are of importance, and it is found that the above formula may be most conveniently applied in its logarithmic form

$$\frac{1}{s}\log q = \frac{\log k_m}{s} + \log r,$$

or

$$\rho = a + b\delta;$$

where

$$a = -\frac{1}{s}\log k_m,$$

$$b = \frac{1}{s},$$

$$\rho = \log r,$$

and

$$\delta = \log q.$$

For each guinea-pig four values of ρ and δ are known, and so a and b may be obtained by the method of least squares: values of k_m and of $1/s$ are thus obtained for each animal. Since $1/s$ is supposed to be common to all the animals, the values of $1/s$ are to be regarded as deviating from the true value as the result of experimental error, and so the mean value is to be taken as approximately correct. From observations on 58 guinea-pigs it was thus found that $1/s = 0{\cdot}55 \pm 0{\cdot}013$ or $s = 1{\cdot}82 \pm 0{\cdot}043$.

It appears that the value of s is fairly close to 2. If s really had the value 2, the law would be $q = k_m r^2$, that is to say, the area of inflammation would be proportional to the quantity of tuberculin applied. As the basis for the subsequent calculations in this paper, it was decided to adopt the value $s = 2$. This value is close to that found experimentally; it is that arrived at theoretically, by assuming that diffusion takes place in two dimensions, and that inflammation occurs at any point provided that the maximum concentration of tuberculin there exceeds a certain threshold value. It should be emphasised that the results reached below would be but insignificantly altered if some other value of s in the neighbourhood of 2 were taken. It is necessary for purposes of calculation to make a definite choice. On strictly theoretical grounds we ought to find the values of a_m and b from the equation $\rho = a_m + b\delta$, where b is common for all animals and a_m differs from one animal to another, so as to give closest agreement with the observed results. We had previously encountered a similar problem in another investigation, and it was there shown that the value of b, obtained rigorously in this way, differed only very slightly from the average value of the various b's obtained by considering each group of

observations separately, as we have done above. Once the value of b has been determined, the best value of a_m is of course readily obtained. For these reasons, the values of k_m were calculated on the assumption that $s = 2$. These values measure the sensitivity of the various animals to a particular tuberculin. Since the absolute strength of the tuberculin is unknown, only comparative estimates of the sensitivity of the various animals can be obtained. On the other hand, if it is assumed that various tuberculins do not act specifically with regard to certain animals, the ratio between the strengths of two tuberculins is readily obtained if we know the k_m values obtained for these two tuberculins on the same animal. Assuming the tuberculins to be equally diluted, the ratio of the k_m values will clearly give the ratio of the strengths of the tuberculins. This ratio ought therefore to be the same for all animals. In practice, however, it is to be expected that as the result of experimental and observational errors the ratio will not be absolutely constant. The various values obtained from the different animals may be regarded as experimental determinations of the true ratio. The derivation of the most probable value of the true ratio from these experimental determinations requires some consideration. The most direct method is to take one of the samples of tuberculin as the standard of unit strength, the experimental determination on the first animal will then give the strength of the unknown sample as $l_1/k_1 = \alpha_1$, where k refers to the standard tuberculin, and l to the unknown. The other animals yield experimental determinations $\alpha_2 = l_2/k_2$, $\alpha_3 = l_3/k_3$, etc. Regarding the α's as a group of experimental determinations, we may take for the most probable value of α, the mean $\bar{\alpha}$ of α_1, α_2, α_3, etc., and also calculate the probable error of $\bar{\alpha}$ in the usual way. On the other hand, the second sample of tuberculin may equally well be regarded as the standard, and the first as the unknown, then the various determinations on each animal of the first sample in terms of the second are $1/\alpha_1$, $1/\alpha_2$, $1/\alpha_3$, etc., or let us say β_1, β_2, β_3, etc. The mean value $\bar{\beta}$ of the β's would then be taken as the most probable value of β. The real value of α is of course the reciprocal of the real value of β; however, in general, $\bar{\alpha}$ is not the reciprocal of $\bar{\beta}$. If all the α's were identical and equal to $\bar{\alpha}$, so that all the β's were identical and equal to $\bar{\beta}$, $\bar{\beta}$ would be equal to $1/\bar{\alpha}$. Roughly the greater the scatter of the α's the greater the divergence between $\bar{\beta}$ and $1/\bar{\alpha}$.

Since there is no *a priori* reason to assume that any one tuberculin has particular claims to be regarded as the standard, a method was sought for, in which the k's and l's were given equal prominence, so that a final ratio could be obtained which was symmetrical with respect to both. A simple method, of doing this would be to take $\beta^* = \sqrt{\bar{\beta}/\bar{\alpha}}$, and $\alpha^* = \sqrt{\bar{\alpha}/\bar{\beta}}$, so that $\alpha^* = 1/\beta^*$.

These values α^* and β^* are in many ways very convenient as measures of the relative strengths of the samples, and it will now be shown that their probable errors may be calculated and put in a form convenient for arithmetical computation.

Let $\alpha_1 = \bar{\alpha} + \alpha_1'$, where α_1' is comparatively small and in general let $\alpha_r = \bar{\alpha} + \alpha_r'$, thus $\Sigma\alpha_r' = 0$. Then

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$$\beta_r = \frac{1}{a_r} = \frac{1}{\bar{a} + a_r'} = \frac{1}{\bar{a}}\left(1 - \frac{a_r'}{\bar{a}} + \frac{a_r'^2}{\bar{a}^2}\right) \text{ approximately}^1.$$

Thus
$$\bar{\beta} = \frac{1}{\bar{a}}\left(1 + \frac{1}{N}\frac{\Sigma a_r'^2}{\bar{a}^2}\right),$$
$$= \frac{1}{\bar{a}}\left(1 + \frac{\sigma_a^2}{\bar{a}^2}\right).$$

Let $1/p = \sqrt{\bar{a}\bar{\beta}}$, and write $1 - p = z$, then

$$p = (\bar{a}\bar{\beta})^{-\frac{1}{2}} = \left[\bar{a}\frac{1}{\bar{a}}\left(1 + \frac{\sigma_a^2}{\bar{a}^2}\right)\right]^{-\frac{1}{2}},$$
$$= 1 - \frac{1}{2}\frac{\sigma_a^2}{\bar{a}^2},$$

then
$$z = \frac{1}{2}\frac{\sigma_a^2}{\bar{a}^2}, \text{ or } \frac{\sigma_a}{\bar{a}} = \frac{\sigma_\beta}{\bar{\beta}} = \sqrt{2z}.$$

Since
$$\sigma_{\bar{a}} = \frac{\sigma_a}{\sqrt{N}},$$

we have
$$\frac{\sigma_{\bar{a}}}{\bar{a}} = \frac{\sigma_{\bar{\beta}}}{\bar{\beta}} = \sqrt{\frac{2z}{N}}.$$

It is to be observed that although $\bar{a}$ is not the reciprocal of $\bar{\beta}$, yet it is so to the first approximation, and to this degree $\bar{a}$ and $\bar{\beta}$ are correlated, with a coefficient of $r = -1$ (approximately).

Hence
$$\sigma_{\beta^{*2}} = \beta^{*2}\left(\frac{\sigma_\beta^2}{\bar{\beta}^2} + 2\frac{\sigma_{\bar{\beta}}}{\bar{\beta}}\frac{\sigma_{\bar{a}}}{\bar{a}} + \frac{\sigma_{\bar{a}}^2}{\bar{a}^2}\right)^{\frac{1}{2}},$$
$$= 2\beta^{*2}\frac{\sigma_{\bar{\beta}}}{\bar{\beta}},$$

or
$$\frac{\sigma_{\beta^{*2}}}{\beta^{*2}} = 2\frac{\sigma_{\bar{\beta}}}{\bar{\beta}} = 2\frac{\sigma_{\bar{a}}}{\bar{a}}.$$

But
$$\sigma_{\beta^{*2}} = 2\beta^*\sigma_{\beta^*},$$

therefore
$$\frac{\sigma_{\beta^*}}{\beta^*} = \frac{\sigma_{\bar{\beta}}}{\bar{\beta}} = \frac{\sigma_{\bar{a}}}{\bar{a}} = \sqrt{\frac{2z}{N}}.$$

Similarly
$$\frac{\sigma_{a^*}}{a^*} = \sqrt{\frac{2z}{N}}.$$

The probable errors of β^* and a^* are therefore $0{\cdot}67\beta^*\sqrt{\frac{2z}{N-1}}$, and $0{\cdot}67a^*\sqrt{\frac{2z}{N-1}}$, respectively.

In the arithmetical application of the above the successive steps are:

From the formula $a = \frac{\Sigma\rho}{n} - b\frac{\Sigma\delta}{n}$ (b being taken as $\frac{1}{2}$ as explained above,

[1] This and the following approximations assume that $\frac{a_r}{\bar{a}}$ is sufficiently small; a condition which in practice is usually fulfilled.

and $\Sigma\rho$ and $\Sigma\delta$ being calculated from the data), the value of a is calculated for each animal with each tuberculin.

Since $$2a = -\log k_m,$$

k_m is readily found, and similarly l_m (for a second tuberculin): thus $\alpha_m = l_m/k_m$ is at once obtained, α_m, l_m and k_m referring to animal numbered m.

The values $\beta_m = 1/\alpha_m$ are next found.

From α_1, α_2, α_3, etc., $\bar{\alpha}$, the mean of these values is obtained, and from β_1, β_2, β_3 the value of $\bar{\beta}$ is obtained.

$$\alpha^* = \sqrt{\frac{\bar{\alpha}}{\bar{\beta}}}, \quad \beta^* = \sqrt{\frac{\bar{\beta}}{\bar{\alpha}}}, \quad p = \frac{1}{\sqrt{\bar{\alpha}\bar{\beta}}},$$

and $z = 1 - p$ are then calculated. The probable error of β^*, the measure of the strength of the second tuberculin in terms of the first is then obtained from the formula

$$0{\cdot}67\beta^* \sqrt{\frac{2z}{N-1}}.$$

Discussion of results.

The methods described above have been applied to six tuberculins each of which has been assayed in terms of the standard supplied by the Medical Research Council. All of these have been estimated by means of observations on guinea-pigs by intradermal application, and four have been carried out on human beings by the percutaneous method. In certain cases a duplicate series of observations was made with the same tuberculin, and in addition 50 and 75 per cent. dilutions of the standard were assayed as controls. The results are summarised in the following table.

Strengths of tuberculins. (Standard = 1.)

	Human test		Guinea-pig test	
	Strength	% error	Strength	% error
B_1	(α) 1·091	6·7	(α) 0·818	3·2
	(β) 1·074	3·4	(β) 0·863	12·5
B_2	—	—	(α) 0·805	7·3
			(β) 0·988	3·7
B_3	—	—	0·888	6·2
C	0·983	14·1	1·667	10·4
D	0·485	6·9	0·819	11·4
E	0·587	6·4	1·074	16·6
Standard diluted:				
0·50 A	—	—	0·541	9·3
0·75 A	—	—	0·741	4·1

Of these tuberculins, B_1, B_2, B_3 refer to separate batches prepared in the same laboratory, C, D and E to samples from other laboratories, and the symbols (α) and (β) denote duplicate determinations on the same batch of tuberculin.

It will be seen that the percentage error of each determination varies in the case of the human experiments from 3·4 to 14·1 per cent., with an average of 7·5 per cent., whilst in the experiments on guinea-pigs the range is from 3·2 per cent. to 16·6 per cent. with an average of 8·5 per cent. From the point

of view of consistency of results there is therefore little to choose between the human and the guinea-pig experiments. These conclusions are confirmed by the duplicate assays of the same tuberculin on the same type of animal, and also by the assay of the known dilutions of the standard.

For example the three pairs of duplicate determinations B_1 (α and β) (human), B_1 (α and β) (guinea-pig), and B_2 (α and β) (guinea-pig), give differences of such a magnitude, that an equal or greater difference would occur, 89 times, 78 times and 7 times in 100. Similarly the results of 54 and 74 per cent. obtained with the known dilutions of the standard are such that equal or greater discrepancies would be obtained in 58 per cent., and 84 per cent. of cases respectively. These figures show that the fluctuations are due to random errors.

When we compare the results of the assay of the same tuberculins on humans and on guinea-pigs, less consistent figures are obtained. For example a difference greater than or equal to that between $B_1(\alpha)$ (human and guinea-pig) would occur only 17 times in 1000, between $B_1(\beta)$ (human and guinea-pig) 20 times in 100, between C (human and guinea-pig) 38 times in 1000, between D (human and guinea-pig) 10 times in 100, between E (human and guinea-pig) 7 times in 100. It seems extremely unlikely that, if the results obtained on the human being and on the guinea-pig were really consistent, a series of such rather improbable discrepancies would be obtained in six consecutive comparisons. This point is of considerable interest in connection with the question as to how far experiments on guinea-pigs are suitable for the assay of tuberculins which will ultimately be administered to human beings. The point merits further study.

SUMMARY.

It will be convenient to summarise the results of the foregoing observations and the conclusions which appear justified by a comparison of results obtained by the two methods of standardisation under consideration, namely (1) cutaneous, on the human subject, and (2) intracutaneous (intradermal) on the guinea-pig.

1. In the absence of knowledge regarding the essential chemistry of tuberculin, a chemical method of standardisation is excluded.

2. The phenomena of allergic reactivity point to a biological basis for standardisation.

3. The allergic reactions have been studied in the sensitised animal and in the human tuberculised subject.

4. Tuberculins of unknown potency have been compared with a tuberculin of known potency which has been selected as standard.

5. Comparative observations have been made as between the standard and the unknown tuberculin, by (*a*) making use of different dilutions, and (*b*) assessing results in animals (sensitised) and in human (tuberculised) subjects.

6. In the sensitised guinea-pig the intracutaneous method has been used, and a definite procedure followed in the determination of results.

7. In the human tuberculised subject the cutaneous method has been used, and similar procedure followed for the determination of results. The method and results are illustrated in the text.

8. The results in the two sets of observations are definite and comparable. Both afford a basis for standardisation.

9. The average probable errors by the two methods are approximately equal, that is, the accuracy of the one method is approximately equal to that of the other.

10. If this be so, a strong plea may be advanced in favour of the human test. Shortly expressed: human (tuberculised) subjects are readily available for observation. The procedure involves little preparation, and the results are easily read with exactness. The tuberculin under test is to be used thereafter in relation to the human subject. This fact enhances the value of the test observations.

If it be objected that in intracutaneous injection the amount of tuberculin introduced is measured more precisely, it may fairly be maintained that the droplet application of tuberculin is limited to a sharply defined area of skin surface and, further, that the clear skin of the human subject allows of more accurate estimation of the diameters of the areas of reaction.

The present enquiry has shown that assays on a variety of human (tuberculised) subjects yield consistent results, and similarly, assays on various animals give consistent results. Yet the animal results are not always contistent with the human results. The explanation of the discrepancy is not very clear. It is not impossible that certain strains of tuberculin act in less degree on the human subject and in greater degree on the animal, and conversely. The occurrence of such differences might be misleading and even involve risk, if standardisation tests were limited to animals without control from observations on the human subject.

If we grant, as the records have shown, that the procedure in relation to the human subject is sound and is innocuous to the human subject of the test, much may be said for the simplicity of the method and for the clarity of results obtained. As the tuberculins under test are destined for use on human subjects —for diagnostic and therapeutic purposes—it would seem reasonable, and probably safer, to base the standardisation of tuberculin (for human purposes) on observations in relation to man.

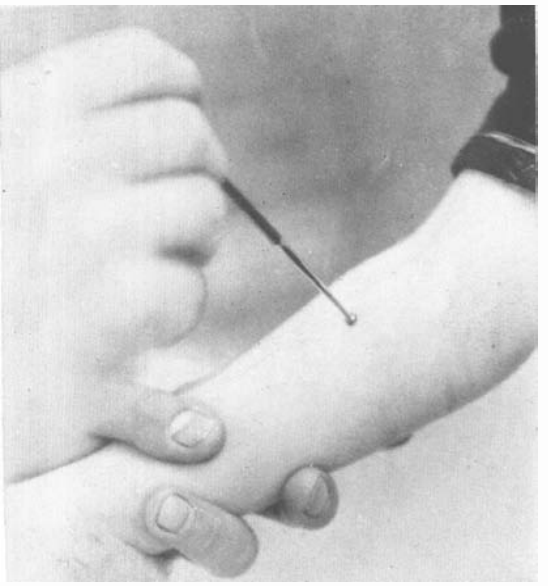

Fig. 1

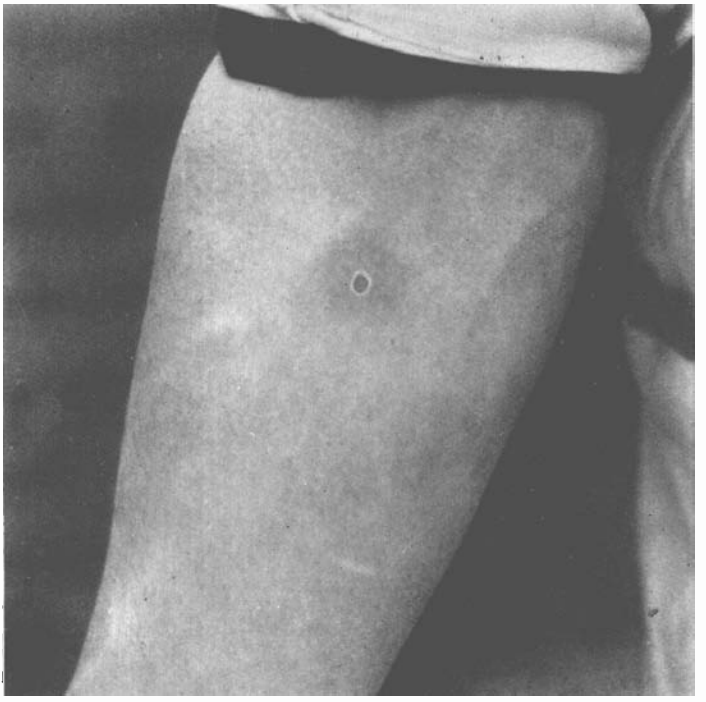

Fig. 2

Figs. 1 and 2. Reprinted from the *Edinburgh Medical Journal.*

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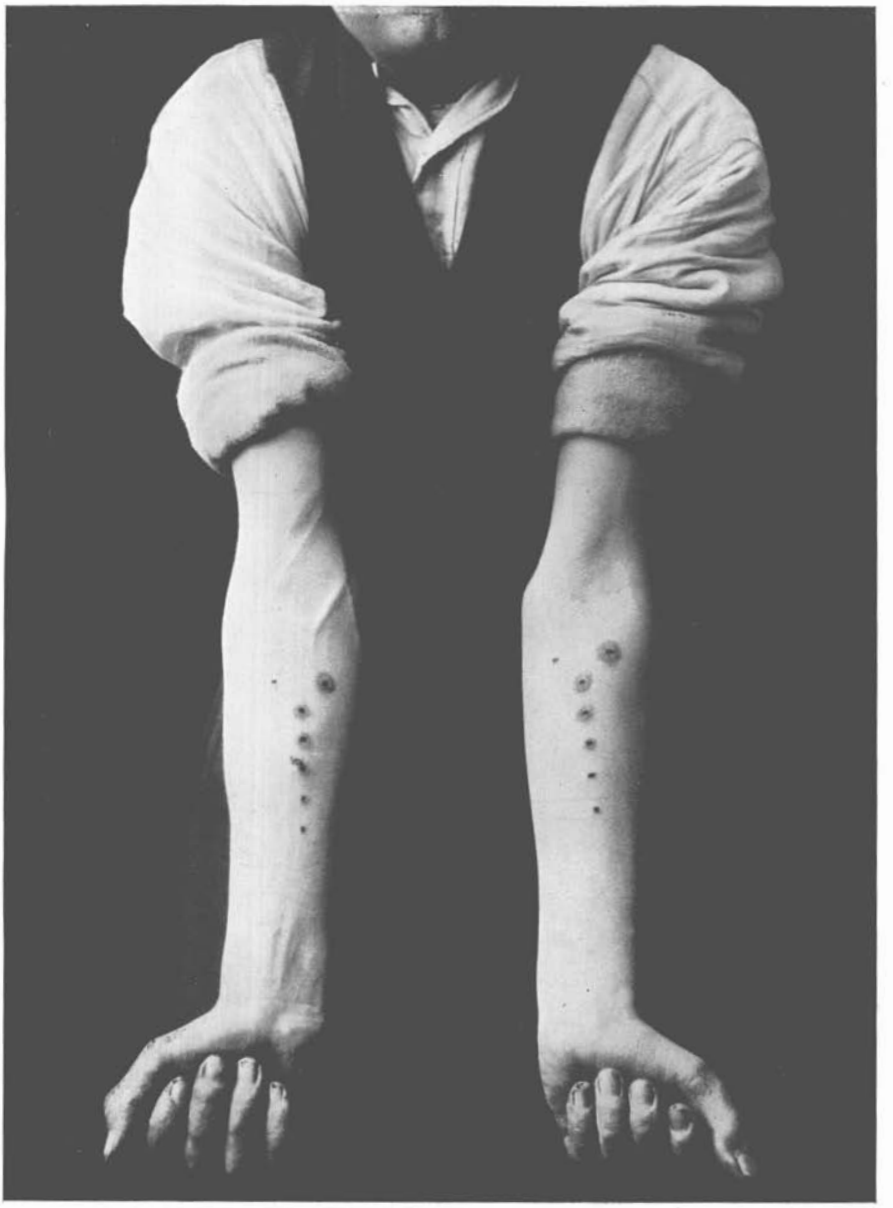

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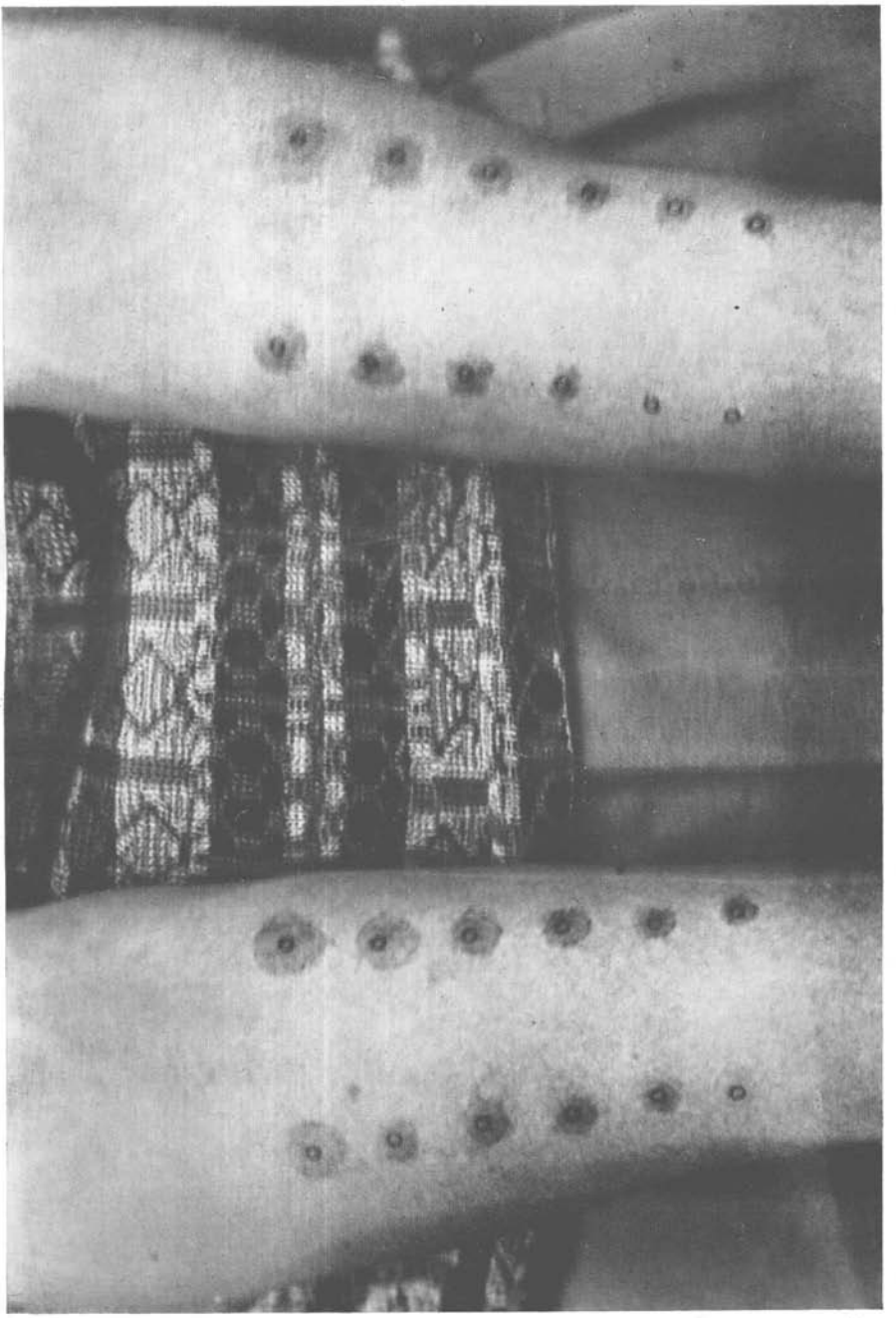

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