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From the Wellcome Physiological Research Laboratories, Beckenham, Kent.

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IN spite of the large amount of work that has been done on the standardization of tuberculin, we have not been able to find in the literature of the subject any adequate study of the accuracy of the tests usually employed, though this is a necessary preliminary to any legitimate claim to be able to standardize the active principle. This, no doubt, is due to the fact that at best the methods available are not very accurate, and have hardly been studied beyond the qualitative stage. In the comparison of a pair of samples of tuberculin the limitations of the tests may not be fully realized.

In the following paper we have endeavoured to collect some data which may help in determining the value of three of the best-known methods of comparison. The three tests investigated were the—

(1) Intradermic (Eagleton and Baxter, 1923),
(2) Von Pirquet (quantitative) and
(3) Subcutaneous (Frankfort, Otto and Hetsch, 1921).

In all these tests the guinea-pig was used as the experimental animal.

With regard to the intradermic and von Pirquet methods our principle was a simple one. A tuberculin of approximately the same value as the Frankfort standard was taken and compared with dilutions of itself in concentrated glycerine veal broth, which were prepared by our colleague, Dr. J. W. Trevan. It was our problem to find out what percentage differences we could detect by the various methods. Each of the dilutions was regarded by us as a tuberculin of unknown strength, and subsequently diluted appropriately in saline for the actual tests. These samples were compared with a sample of the undiluted tuberculin which was used as the standard for each experiment. A sufficient number of animals was injected so that the results would be capable of numerical analysis. After each experiment the estimated values were compared with the actual values as then supplied by Dr. Trevan.

TECHNICAL DETAILS.

The technical details of the work were on the lines indicated by Eagleton and Baxter, and need only be referred to briefly. Guinea-pigs of 300 to 350 gm. in weight were inoculated subcutaneously with 0·5 mgm. or 0·25 mgm. of a three weeks' growth of the human strain of *B. tuberculosis* (H. 12) on Dorset's medium. With this inoculum, guinea-pigs were appropriately sensitive to the intradermic and von Pirquet test in about three weeks, and to the subcutaneous test in six to eight weeks. For the intradermic test they were sensitive on an average to an injection of 0·2 c.c. of a 1 in 2000 to 1 in 4000 dilution of standard tuberculin. In the von Pirquet test they reacted to 1 in 4 to 1 in 8 of standard tuberculin. For the subcutaneous test

the guinea-pigs were sufficiently sensitive that 0·1 c.c. of standard tuberculin (= Frankfort standard) killed about 50% of the guinea-pigs tested.

The series of dilutions used in the intradermal test were usually 1 in 500, 1 in 1000, 1 in 2000 and 1 in 4000 of the preparations under test, and the amount injected 0·2 c.c. In the von Pirquet test the tuberculins were generally used undiluted, and also diluted 1 in 4, 1 in 8, 1 in 16 and 1 in 32; three parallel scratches, half an inch long, were made for each dilution. The readings were made 18 to 24 hours after injection in the case of the intradermic and von Pirquet tests. Usually the reactions were matched or graded by eye, but during part of the work measurements were taken by means of calipers, and the results almost exactly agreed with the matching by eye.

INTRADERMIC TEST IN GUINEA-PIGS.

Calmette and de Potter (1926), in their recent report on methods of preparing and standardizing tuberculin, came to the conclusion that this is the most useful method of titration. In our hands it has also proved the most valuable method.

TABLE I.—*Actual and Estimated Values of Samples of Tuberculin Tested by the Intracutaneous Method.*

Sample.	Correct values.	Estimated values.	Number of animals used.	Number of animals readable.
A	100	100	13	11
B	50	45		
C	50	55		
D	100	75		
E	100	100	10	10
F	60	70		
G	50	60	10	7
H	70	60		
J	70	65	9	7
K	40	30		
L	60	65	14	9
M	30	40		
N	70	100	9	5
O	25	35		
P	0	0	6	5
Q	10	15		
R	0	0	7	6
S	75	80		
T	10	5–20 (probably 8–10)	10	7
U	15	5–20 (probably 8–10)		
	Total		88	67

An initial attempt was made to place samples of tuberculin of different values and the standard tuberculin in their correct order. This was done with 5 series of 4 tuberculins, 50 guinea-pigs being used for the experiments. If the tuberculins differed 40% or more in potency, it was nearly always possible to place them in their correct order by the test.

Taking 40% as the difference between two tuberculins that was just clearly detectable, a further series of tuberculin preparations was compared and percentage values allotted to them (Table I). The values estimated from the test are not to be taken as more than attempts to assign a percentage, but it will be seen that the attempts were almost always good ones. Only once in 20 comparisons was the estimated value further out than 30%, and it was usually a good deal less. Considerable numbers of guinea-pigs had, however, to be used in order to arrive at this degree of accuracy, and it must be clearly understood that the reading of individual guinea-pigs may give values that are widely aberrant. For practical purposes 4 to 6 guinea-pigs will indicate differences of 40 per cent. with a fair degree of certainty, and with the use of a greater number of animals considerably finer differences may be detected. It may be added that the average standard deviation of the series of values given in Table I corresponds to an error of 22·2 per cent. of the activity of the sample tested—that is, the expected error would be rarely above 44·4%, which agrees with the rough estimate first arrived at.

QUANTITATIVE VON PIRQUET TEST IN GUINEA-PIGS.

Table II gives the results of 16 comparisons of tuberculin preparations with the standard tuberculin. It will be seen that this method gave much less satisfactory results than the intradermic method. Occasionally good estimates were made, but in many cases the estimate was very inaccurate. The chances of being able to detect 50% differences are small, and it is possible to make such a gross error as to state that a given sample is 50% of standard strength when in reality it is tuberculin-free (*e.g.* sample K1). With weak tuberculins, also, very wide errors can be made, as the maximum reactions produced on the guinea-pigs may not be good enough to be read with ease. On individual guinea-pigs the reactions due to standard tuberculin, undiluted, and diluted 1 in 4 and 1 in 8, may all be indistinguishable. To get the maximum accuracy with this test the tuberculins and their dilutions must give well-marked and well-graded reactions.

To sum up, if the tuberculins are sufficiently strong, the guinea-pigs highly sensitive, and the gradation of the reactions good, 50% differences are often detected by the von Pirquet test, but such wide errors may be made that the test may be considered as useless for the purposes of tuberculin standardization.

SUBCUTANEOUS TEST IN GUINEA-PIGS.

The figures for this test were arrived at by a somewhat different method. As each batch of guinea-pigs became appropriately sensitive, it was subdivided into four groups, which were injected, not with unknown dilutions, but with

TABLE II.—*Actual and Estimated Values of Samples of Tuberculin Tested by the von Pirquet Method.* (*S = Standard Tuberculin.*)

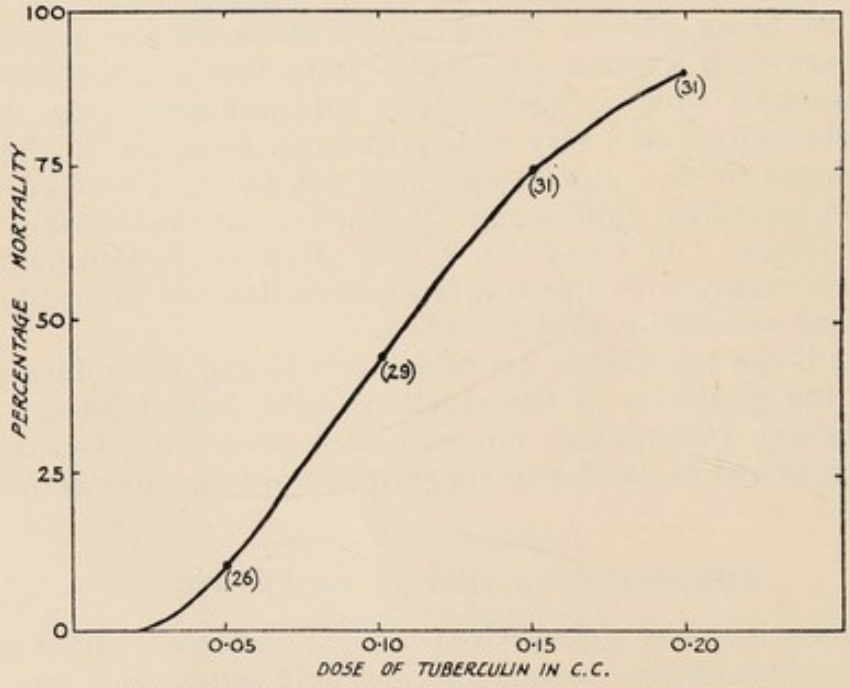
Sample.	Correct values.	Estimated values.	Number of animals used.	Number of animals readable.
V	70	100	5	4
W	80	<S		
X	50	<S<S<S=S*	9	4
Y	20	=S<S=S<S		
Z	27·5	35	7	7
A_1	10	25		
B_1	16·6	33·3	6	5
C_1	50	33·3		
D_1	0	25	5	5
E_1	50	25		
F_1	90	90	5	5
G_1	50	70		
H_1	50	25	5	5
J_1	25	12		
K_1	0	50	7	5
L_1	100	50		
Total			49	40

* Readings on individual guinea-pigs.

CHART I.

STANDARDISATION OF TUBERCULIN – SUBCUTANEOUS TEST.

PERCENTAGE MORTALITY IN RELATION TO DOSE.



THE FIGURES IN BRACKETS SHOW THE NUMBER OF ANIMALS INJECTED WITH EACH DOSE

0·05, 0·1, 0·15 and 0·2 c.c. of standard tuberculin. Chart 1 represents graphically the percentage mortality in guinea-pigs in relation to increasing doses of standard tuberculin (that is to say, in relation to the actual amount of active principle present). The figures on the curve indicate the number of guinea-pigs put up at each dose. They represent several batches of tuberculous guinea-pigs, but the batches were, as far as could be arranged, of equivalent sensitiveness. From this curve it can be calculated that with two groups of eight animals, tuberculins in an activity ratio of 2 to 1 (*i.e.* a drop of 50%) will not be placed in the wrong order more than once in 21 times. On this calculation it would require 25 animals in each group to differentiate a sample of tuberculin from one 30% less potent.

CHART II.

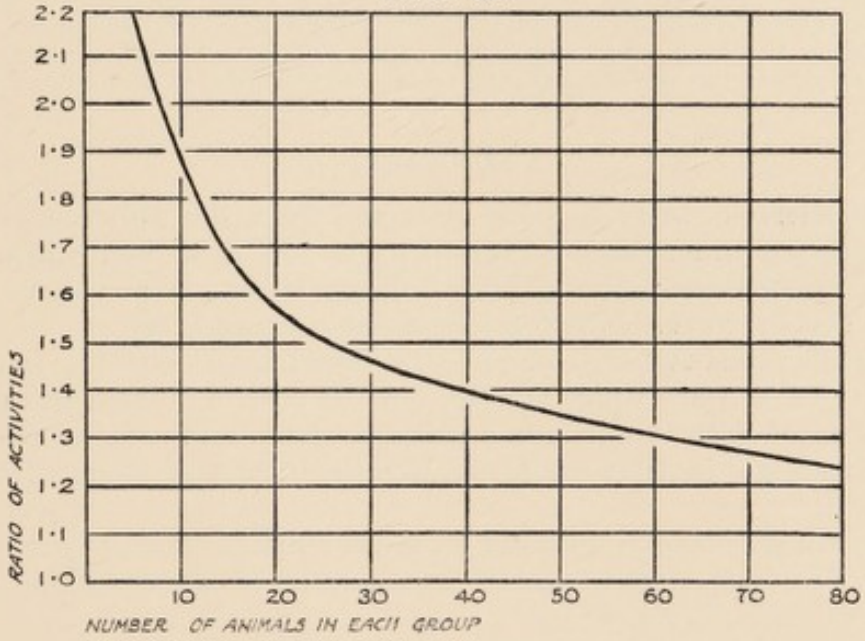


Chart 2, kindly constructed for us by Dr. Trevan, utilizes the same data to show the number of animals required to obtain a given degree of accuracy. Thus to distinguish 20 times out of 21 a tuberculin of value 1·4 from a tuberculin of value 1 it would require 40 guinea-pigs for each tuberculin, and so on.

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

The curve (Chart 2) shows approximately the various ratios of the doses of tuberculin which are sufficient to give a significant difference in mortality using various numbers of animals. The number of animals injected with each dose is given by the abscissæ, and the ratios given by the curve are the ratios corresponding to twice the standard deviation of the difference of the mortality in the two groups. The ratios given therefore represent doses of which the smaller will give a mortality less than the larger, in 20 cases out of 21 on the average. The curve has been calculated for the cases in which the doses administered happened to be such that the mortalities are symmetrically disposed around 50% mortality. For other pairs of mortalities the ratio giving a significant difference may be slightly different. The values of the ratio for 40 animals, for example, may fluctuate between 1·36 and 1·56. The curve was constructed in the following manner:

Let S be the standard deviation of the difference of mortality in the 2 groups; then

$2S$ represents a significant difference. If the doses are such that the mortalities are symmetrically disposed around 50 %,

$$S = 100 \sqrt{\frac{2p\,(1-p)}{N}}$$

expressed as % mortality, where p is the probability of death with the smaller and survival with the larger dose and N is the number of animals in each group and—

$$\frac{100-2S}{2} = 100p,$$

and from this it can be shown that—

$$p = \tfrac{1}{2} \pm \sqrt{\frac{1}{2N+4}}.$$

$100p$ is the percentage rate of mortality given by one dose and $100\,(1-p)$ is the percentage rate of mortality just significantly different. The doses which correspond to these mortalities are read from the mortality curve (Chart 1) and the ratio calculated for different values of N.

It is of course clear that all these calculations depend on the accuracy with which the curve (in Chart 1) represents the true relationship between mortality and dose. The number of observations is not large and there is a possibility of error in the estimates of the ratios calculated from it, but the calculations give an idea of the order of accuracy of the test.

SUMMARY.

The intradermic test is more accurate than the subcutaneous or von Pirquet methods, differences representing a 40% drop being almost always detectable on a small series, and finer differences on a larger series of guinea-pigs. The test, however, calls for considerable experience.

The subcutaneous test will also show differences of 40–50%, if a sufficient number of suitable guinea-pigs is used, but the cost in guinea-pigs of attaining a given degree of accuracy is much greater than with the intradermic test. Its one advantage is that there is practically no personal factor in its interpretation.

The von Pirquet test in guinea-pigs is very inaccurate and gross errors are often made. It is practically useless as a method of standardizing tuberculin.

We have to thank our colleague, Dr. J. W. Trevan, for much help and criticism in this work.

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