presented similar findings under the same condition. In an additional group of 9 females put on the diet after maturation, only 2 showed the large cells described above. The reproductive system was examined in all animals. The rats which were 2½ to 3 months of age and had been on the diet 2 months or less showed a retardation of the reproductive organs. Among the males who remained on the diet for a longer period, 11 showed only slight retardation on histologic examination; one appeared definitely retarded. The females reared on the diet showed delayed maturity at the age of 5 or 6 months, while those put on the diet after maturation presented normal reproductive organs. In order to rule out the absence or insufficiency of vitamins E and A as the cause for the changes found, wheat germ oil and carotene oil were added to the diet of some of the experimental animals. These additions had no effect upon the results.

10095

The Standardization of Diluted Tuberculin.

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In developing the method for standardization of tuberculin described in this paper, we were seeking one superior to any hitherto reported, in two respects: (1) a method that would be accurate within at least 20% instead of the 40 and 60% accuracy hitherto available; (2) an accurate method so sensitive that it could be used to standardize tuberculin ready diluted 1-10,000. We required such a method so that tuberculin ready diluted in the diluent previously described[1] could be tested with precision after dilution, and just before distribution, so that the material used by the physician in the field could be of known potency.

We had been comparing tuberculin of our manufacture with the International Standard tuberculin of the League of Nations for a number of years by injecting a 1-10,000 dilution of the standard and the unknown tuberculin in each arm of 100 individuals. Variations in the results indicated that this was far from an accurate method of comparison. Also carefully run experiments in which a 1-10,000 dilution, and 70, 80, or 90% of this concentration of a

[1] Gottschall, R., and Bunney, W. E., *J. Immunol.*, 1938, **34**, 103.

tuberculin equivalent to the International Standard, were injected into the 2 arms of sensitive individuals, showed that a 20% difference in 2 dilutions could not be determined reliably by this method. These injections were made at the Ingham Sanatorium, Lansing, by Dr. G. C. Stucky, resident physician, to whom we are indebted for this part of the work. The injections were made as unknowns and read independently by 2 workers as unknowns. As much difference was found between the 90 and 100% reactions as between the 70 and 100%, and the 80% reactions averaged larger than the 100%.

The use of guinea pigs as test animals has advantages. They are more readily available and multiple injections can be used, so that a series of dilutions of 2 tuberculins can be compared on the same animal. The method of testing diluted old tuberculin used by Parish and Okell,[2] and by Douglass and Hartley,[3] in their studies on the properties and stability of "synthetic" old tuberculin was found to be inadequate for testing first test-dose material (1-10,000 dilution), since too few of their animals were sensitive enough to react satisfactorily to a 1-10,000 dilution of tuberculin. With their method we found but 2 of 15 animals to be suitable for titrating 1-10,000 tuberculin. The method eventually found most satisfactory is a modification of that devised for the standardization of old tuberculin on tuberculous guinea pigs as described by Lewis and Aronson[4] and Eagleton and Baxter.[5] This method was suggested by Calmette and de Potter[6] as the most valuable for standardization of tuberculin, and as modified appears to fulfill the requirements we had adopted as our goal.

Sensitization of Animals. White 700 to 1400 g guinea pigs, from 6 to 24 months old, are sensitized by injecting in the groin, 0.5 mg (wet weight) of a 4-week culture of Saranac Lake strain of *Myco. tuberculosis,* H37, grown on Dorset's synthetic agar medium, or Petragnani's medium. Old healthy discarded breeders or animals which have been released from other biological tests are particularly well suited for this work. The suspension of organisms is prepared by thoroughly triturating the weighed bacilli in a tube by means of a glass rod, adding a drop of warm liquefied gelatin and emulsifying until an homogeneous mixture is obtained. Sufficient sterile Clark and Lubs phosphate buffer, pH 7.2 to 7.4 is slowly added with constant stirring to make a final concentration of 1.0 mg per milliliter.

2 Parish, H. J., and Okell, C. C., *J. Path. and Bact.*, 1929, **32**, 51.

3 Douglass, S. R., and Hartley, P., *Tubercle*, 1934, **16**, 105.

4 Lewis, P. A., and Aronson, J. H., *Am. Rev. Tuberc.*, 1923, **7**, 404.

5 Eagleton, A. J., and Baxter, E. M., *Brit. J. Exp. Path.*, 1923, **4**, 289.

6 Calmette, A., and de Potter, *Ann. de l'inst. Pasteur*, 1926, **40**, 353.

The animals are sufficiently sensitive at the end of 4 weeks and are then tested by injecting intracutaneously 0.1 ml of a 1-10,000 dilution of a standard tuberculin. Those showing a 2+ reaction (10 to 20 mm induration) 48 hours later are used for testing old tuberculin at the 1-10,000 level. We have found that at least 90% of the animals sensitized in this manner can be used for titrating 1-10,000 tuberculin and some of our animals have reacted to a 1-320,000 dilution of a tuberculin equivalent to the International Standard. Parish and O'Brien[7] found the "sensitivity limit" of their animals to be 1-8000 but several of their animals reacted to a 1-25,000 dilution of tuberculin equivalent to the International Standard.

Description of the Test. 1-20,000, 1-40,000, and 1-80,000 dilutions of tuberculin are prepared in saline from the first strength or 1-10,000 dilution of the unknown to be tested for potency. A similar series of freshly prepared dilutions from 1-10,000 to 1-80,000 and another series at 80% of these values or 1-12,500, 1-25,000, 1-50,000, and 1-100,000 are made. One-tenth ml of these 12 dilutions are injected across the back[5] of 3 closely clipped animals, each series being placed at a different position on each animal to avoid as far as possible variation in size and intensity of reactions due to location alone. The highest dilution of each series is always placed on the left side farthest from the backbone and the other dilutions in order across the back. The injection-scheme is as follows:

Guinea pig 1	Guinea pig 2	Guinea pig 3
100% series	Unknown	80% series
80% "	100% series	Unknown
Unknown	80% "	100% series

The skin is first marked off with a wax pencil, the first series being placed well behind the nape of the neck and the last series in the lumbar region. Reactions on the nape of the neck or too far toward the rump of the animal are frequently unreliable. When the second test dose or 1-100 dilution is titrated this tuberculin and the standard are also diluted to the 1-10,000 level and injected as in titrating the 1-10,000 dilution.

After 24 and 48 hours the reactions are read and an attempt is made to differentiate the 100% from both the 80% and the unknown series before locating their positions from the records. If the 100% series has been correctly differentiated from the unknown or the 80% series the assumption is made that a difference of 20% is readable on this animal and the unknown is compared with the 80 and 100% series and read as being 100%, 80 to 100%, or less than 80%

[7] Parish, H. J., and O'Brien, R. A., *Brit. Med. J.*, 1935, **1**, 1018.

of the standard. In case the 80% and 100% reactions are similar but the unknown reactions are weaker the value of the unknown must be less than 80%. Those animals in which all 3 series are similar are discarded as unreadable since a variation in strength of 20% is not distinguishable. Readings are made 24 and 48 hours after injection.

One-ml tuberculin syringes and 28-gauge needles are used for making injections. Because of the "tenacity" of tuberculin[7] the syringes are treated with strong chromic-acid cleaning solution for 2 to 4 days to remove traces of tuberculin not destroyed by ordinary cleaning as are also the Bureau of Standards pipettes used for making the dilutions.

Occasionally animals on preliminary test with 1-10,000 tuberculin show an area of induration of 20 to 30 mm diameter, with a central necrotic area. It is then necessary to use dilutions either from 1-20,000 to 1-160,000, or 1-40,000 to 1-320,000 (depending on the sensitivity) instead of 1-10,000 to 1-80,000. Large reactions do not appear to give as fine a differentiation as smaller reactions. The highest dilution should give a diameter of redness of from 4 to 8 mm if the test is to be accurate.

This method has been found valuable for routine testing of ready-diluted tuberculin before distribution, and for following the stability of diluted and undiluted tuberculin. It seems to be more accurate than the method in common use in which Okell and Parish[8] found a difference of 40% between 2 tuberculins to be just barely differentiable.

After the completion of this work it was thought desirable to determine the accuracy of the tests by titrating a number of unknown percentages of 1-10,000 dilutions of tuberculin. Ten out of 50 percentages of 1-10,000 dilutions ranging from 60 to 100% of the standard 100% or 1-10,000 dilutions were therefore selected by a disinterested worker and tested by one of us who was ignorant of the values of the tuberculin. The tests were made in the manner previously described, 3 animals being used for each unknown. Results are shown in Table I. While the number of unknown titrations is small it is apparent from these figures that the assigned values were all within 20% of the actual values.

We feel safe in assuming that a 1-10,000 dilution of tuberculin which has lost potency can be detected if the loss is greater than 10%, and that the actual loss can be estimated correctly within 20%. The use of a larger number of animals for each titration would prob-

[8] Okell, C. C., and Parish, J. H., *Brit. J. Exp. Path.*, 1927, **8**, 170.

TABLE I.
Actual and Assigned Values of Unknown Dilutions of Tuberculin. Diluted 1-10,000

Unknown No.	Actual value of unknown	Assigned value
1	70%	70-80%
2	70	60-70
3	80	90
4	100	90
5	60	70
6	80	90
7	90	90
8	90	80-90
9	80	70-80
10	70	70-80

ably still further increase the sensitivity of the test.

In the development of the test it was found essential to compare a series of dilutions of the 2 tuberculins as described so that the series of dilutions could be evaluated as a whole. This because in our hands it was impossible to differentiate with assurance between 2 dilutions differing from each other by 30 or even 40%, if single injections of each dilution were made in the same guinea pig. As a corollary of this fact it was found that multiple injections of a single dilution into some guinea pigs could give reactions varying as much as 40% in size. Not until we used a series of dilutions causing a 2+ reaction in the lowest dilution, and a diameter of redness of 4 to 8 mm in the highest dilution, was an accurate comparison of 2 tuberculins possible, and then only if the 2 series of reactions were compared as a whole.

The minimal number of 3 guinea pigs was adopted for the test because it was found in a series of comparisons on 54 guinea pigs, that two-thirds of them gave readily differentiated reactions to 1-10,000 dilution and 80% of this value. In most of the remaining third the reactions to the 2 dilutions were identical. If a minimum of 3 pigs previously proved sensitive to the 1-10,000 dilution is used it practically never happens that all 3 animals are unsuitable for differentiating a 20% difference in potency.

Summary. A skin-test for determining the potency of diluted tuberculin is described. It can be used to accurately standardize tuberculin ready diluted to the 1-10,000 level. With this method the results are accurate within 20%, consequently a diluted tuberculin which is but 80% of the original strength can be detected with little difficulty. The method is inexpensive since discarded animals can be used without altering the sensitivity of the test. The lower limit of accuracy seems to be well within the limit of error of routine human group-testing.