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## Estimation of Biological Activities of Purified Tuberculin by the Hopkins Tube.

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Since the protein of *Mycobacterium tuberculosis* is now considered to be the active principle responsible for the allergic reaction in the Mantoux intracutaneous test, the dry weight of this substance has been employed as a basis of measuring biological activities on the tacit assumption that all preparations by the same method yield products of the same potency. Recent findings<sup>1, 2</sup> including observations of our own<sup>3</sup> have shown that such a belief is no longer tenable. Indeed, under certain conditions<sup>4</sup> the activity of the substance may be largely destroyed without any material alteration in its dry weight. Since the conditions responsible for these fluctuations in potency are not well understood it may seem obvious that every preparation requires careful standardization. The only satisfactory method for this purpose, according to Seibert and DuFour,<sup>2</sup> is the sensitive human skin, although sensitive guinea pigs may be used for preliminary titrations. Even the human skin does not always remain uniform in its reaction to tuberculin. For example we found<sup>3</sup> that little or no difference could be detected among the following strengths of purified tuberculin when comparative Mantoux tests were performed on 15 human subjects: 0.000,04, 0.000,02, 0.000,01 mg. Similar results were also found by Seibert and DuFour.<sup>2</sup> Apart from this limitation of the tissue-reaction, the expense, time, and technical difficulties involved would seem to warrant a search for a more practical method. In the present communication an attempt was made to determine whether a precipitative method would fulfill the requirements.

*Methods and Material.* Purified tuberculins of various strength were employed for the present study. The details of the preparation will be reported elsewhere. In addition, a PPD prepared by a new method<sup>5</sup> and kindly supplied by Dr. Seibert was used as the stand-

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<sup>1</sup> Jensen, K. A., Bindslev, G., Moller, S., Hansen, A., and Lind, P., *Tubercle*, 1938, **19**, 386.

<sup>2</sup> Seibert, F. B., and DuFour, E. H., *Am. Rev. Tuberc.*, 1940, **41**, 471.

<sup>3</sup> Unpublished results.

<sup>4</sup> Wong, Sam C., and Ouyang, G., *PROC. SOC. EXP. BIOL. AND MED.*, in press.

<sup>5</sup> Seibert, F. B., *J. Biol. Chem.*, 1940, **133**, 593.

ard. Comparisons of biological activities were done on human subjects employing the same precautions, the dosage of tuberculin, the interval of reading, and the site of injections previously emphasized.<sup>4</sup> The volume of precipitate in a given solution was measured in the Hopkins tube as follows: One cc of a clear solution of purified tuberculin was delivered with a volumetric pipet into a Hopkins tube, an equal volume of 25% trichloroacetic acid was added, the mixture was refrigerated overnight, and then centrifuged at 2300 r.p.m. for 30 minutes. A preliminary reading was first taken and the precipitate was tightly packed with a stiff wire. The centrifugation was repeated to ensure that a constant reading has been obtained. It was found that one centrifugation usually suffices. A duplicate sample was always run and the average results of the readings were taken as the value. However, if the difference in readings was greater than 0.002 cc, the whole procedure was repeated employing fresh samples. It is to be especially noted that all solutions employed for this test must be perfectly clear.

*Relation Between Weight and Volume.* In the first experiment we attempted to correlate the dry weight of various preparations of purified tuberculin and the volume of precipitate. For this purpose we employed 4 different batches of our tuberculin together with Seibert's PPD. The nitrogen-contents of the former preparations were equal, 13.27% being found, while that of the latter, according to Seibert,<sup>5</sup> was 16.3%. The results are presented in Table I.

All readings except those of PPD were the average of 4 different determinations. In case of the latter only duplicate samples were run because of lack of material. From the data it is clear that a definite relation exists between weight and volume of the same preparation and that volumes of precipitate vary with different preparations when similar weights are compared.

*Relation Between Biological Activity and Volume.* Since the dry weight of purified tuberculin can not be employed as an absolute measurement of Mantoux-activity as emphasized elsewhere in the report and since the volume of precipitate varies with different preparations of purified tuberculin it is of interest to determine

TABLE I.  
Relation Between Weight and Volume of Precipitate of Various Tuberculins.

Wt in mg	Hopkins tube reading in cc				
	Lot A	Lot B	Lot C	Lot D	PPD
5	.020	.020	.010	.010	.023
2½	.011	.010	.005	.005	.013
1	.004	.004	.003	.002	.005

whether there exists a relation between activity and volume. It was found by careful titrations in the human skin that the various tuberculins presented in Table I possessed the following relative potencies: Lots A and B were equal, lots C and D were half as strong as lot A, and PPD was the strongest. When these values are compared with the volumes of precipitate given in Table I, two points may be emphasized: In the first place tuberculins of the same activity, such as lots A and B gave similar volumes of precipitate while those of different activities yielded different values. In the second place lots C and D were found to be half as strong as lots A and B, their volumes of precipitate were only half of that of the latter. Similarly, PPD was stronger than lot A, its value was greater than the latter. In the light of these findings it should be possible to adjust other preparations of purified tuberculin to Seibert's standard employing the following equation:

$$\frac{\text{Reading in Hopkins tube} \times \text{Constant}}{\text{cc taken}} = (n) \text{ mg per cc}$$

n being the value of the activity of a given solution of purified tuberculin in terms of PPD. Since the volume of precipitate and the weight of PPD are known the same equation may be applied to compute the value of the constant, the average of which is 203.

*Application.* In order to demonstrate the usefulness of the above equation we proceeded to compute the biological activity of our tuberculin, lot A, in terms of PPD, employing the Hopkins-tube values recorded in Table I. Thus a 1% solution when computed

TABLE II.  
Showing Size of Reaction.

Subject	Reading in mm	
	PPD (Seibert's)	Lot A
CKM	20 x 20	20 x 20
LCT	17 x 20	20 x 20
LTH	23 x 23	24 x 25
LLC	16 x 20	18 x 20
SS	25 x 25	25 x 25
LPS	15 x 15	15 x 15
PKL	20 x 20	20 x 20
TTC	25 x 25	25 x 27
WCS	15 x 16	15 x 15
YMS	10 x 10	10 x 10
LKS	15 x 15	15 x 15
CKP	20 x 20	20 x 20
KCL	0 x 0	0 x 0
LPH	0 x 0	0 x 0
WTC	0 x 0	0 x 0

was found to be only 0.8%. When this solution was diluted according to the latter value and compared with Seibert's standard in human subjects complete agreement of Mantoux activities resulted. The data shown in Table II are self-explanatory.

In a similar manner we have applied the equation for estimating the biological activities of a large number of preparations. These included solutions of known concentration but of different activities and solutions prepared by dissolving at random tuberculin-powder in buffer solution. The results of these findings showed consistent agreement of biological activities between Seibert's PPD and our various preparations of purified tuberculin. Incidentally it should be emphasized that the equation employing the constant 203 is not applicable under the following conditions: unpurified heated or undenatured tuberculins, and purified tuberculins hydrolyzed by alkali. The most accurate reading lies between 0.01 to 0.03 cc, while the least is below 0.005 cc. In the former an error of 20% may occur, while in the latter about 50%.

*Comment.* The Hopkins-tube method described above is not new, Seibert<sup>6</sup> has made use of the same in the standardization of synthetic medium tuberculin. It has not, however, been applied in correlating biological activity of various preparations of purified tuberculin. Why tuberculins of the same weight and the same nitrogen content but differing in biological activities yielded different volumes of precipitate is not clear. The possibility that a part of the tuberculin of the less active preparation is not precipitated by the trichloroacetic acid is rendered unlikely by the fact that the original tuberculin represents that portion precipitated by the acid. It seems plausible, however, that under the conditions of the experiment less active preparation gave a more compact precipitate than the active preparations.

Since the method has been applied to purified tuberculins prepared from only one strain of *Mycobacterium tuberculosis* (H37), we believe it is premature to suggest its use for the standardization of all purified tuberculins. Still we believe that the results so far shown indicate that such a method is useful for preliminary estimation of tuberculin of unknown activity or of tuberculin whose weight is not known.

*Conclusion.* On the basis of the above results and subjected to the limitations mentioned it is justifiable to conclude that the Hopkins-tube method is useful for the estimation of biological activity of various preparations of purified tuberculin.

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<sup>6</sup> Seibert, F. B., *J. Biol. Chem.*, 1928, **78**, 345.