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Comparison of Photometric and Weight Estimation of Mycobacterium Content in Homogeneous BCG Cultures containing Tween 80

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A comparison of the weight and photometric methods of primary assay of BCG vaccine has been made, using a vaccine prepared in albumin-free medium but containing Tween 80. In the weight method, the bacteria were trapped on a membrane filter; for photometry a Pulfrich Elpho photometer and an instrument of Czech origin were used. The photometric results were the more precise, provided that the measurements were made within two days of completion of growth; after this time the optical density of the suspension began to decrease slowly. The lack of precision of the weighing method is probably due to the small weight of culture deposit (which was almost on the limit of accuracy of the analytical balance) and to difficulties in the manipulation of the ultrafilter.

Estimation of mycobacteria in BCG vaccine by weight is not without its difficulties. Estimation in terms of moist weight is somewhat inaccurate; dry weight is a more precise figure, but its determination is laborious. We have therefore attempted to develop a photometric method, and have compared its accuracy with that of dry-weight estimation. The work was carried out on a BCG culture growing homogeneously in Dubos medium with Tween 80 but without albumin.

The use of submerged homogeneous BCG cultures for the preparation of vaccine, as suggested by Dubos & Fenner (1950), has pronounced advantages over the classical method. In earlier reports, Galliová et al. (1957, 1958) described their experiences in maintaining the BCG strain in the Dubos medium with Tween and without albumin and the results they obtained in the intradermal administration of the vaccine to human subjects. Despite the fact that even culture growth results from equal inocula, it was considered desirable to develop as exact a method as possible for estimating the weight content of the BCG bacteria in the culture.

No reliable photometric method of estimating the weight content of bacteria in homogeneous cultures has so far been recommended in the literature. Ungar et al. (1956) used the Spekker photometer; however, their paper does not give any technical details. The method recommended in the *International Tuberculosis Yearbook* (1955) is not accurate and is unsuitable for use in vaccine preparation. So far no comparison of dry-weight and photometric estimations has been described.[5]

MATERIALS AND METHODS

Mycobacterium

Strain BCG 725 was used.

Medium

The medium was prepared by heating 6.3 g disodium hydrogen phosphate, 1.0 g potassium dihydrogen phosphate and 2.0 g asparagine in 100 ml water until dissolved. Then 850 ml distilled water, 30.0 ml enzymatic hydrolysate of casein, 0.05 g ferric ammonium citrate and 0.01 g magnesium sulfate septahydrate were added and the volume adjusted to

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[5] After this work was completed our attention was drawn to a published comparison of *wet* bacterial weight and photometric methods (*Handbook of tuberculosis laboratory methods*, 1962).

1000 ml and the pH to 6.5-6.8. Before autoclaving, 5 ml of a 10% solution of Tween 80 were added. The medium was then distributed into 100-ml flasks and autoclaved for 30 minutes at 120°C.

Incubation

The material was incubated for 14 days at 37°C. The growth was diffuse, with a sediment at the bottom. Under the microscope most of the bacteria were seen in small, net-like clusters; large clusters were rather rare. Non-acid-resistant forms were regularly found among the cultures.

Weighing method

A membrane filter (Göttingen, type TBC), 30 mm in diameter, was immersed in 30% ethyl alcohol for 24 hours. It was dried at room temperature and then in a desiccator over phosphorus pentoxide and weighed. Through this filter, 10 ml of a 14-day BCG culture were filtered. Microscopical examination of centrifuged filtrate showed that the membrane filter retained all the bacteria. The filter was rinsed with 15 ml distilled water, dried in a desiccator over P_2O_5 for 72 hours and weighed. The difference between the two weighings gave the weight of mycobacteria per 10 ml of cultivation medium.

Photometric method

Photometry was carried out on a Pulfrich photometer with an S 57 filter, using 1-cm cuvettes; some measurements were performed on this apparatus fitted with the Elpho attachment. A photoelectric photometer of Czechoslovak origin (single-cell, produced by Laboratorní přístroje) was also used.

RESULTS

The stability of the suspension of mycobacteria in the Dubos medium was tested photometrically. Five different 14-day samples of the same culture, all grown under identical conditions in a single batch of medium, were measured on four consecutive days. Between the measurements the cultures were kept in the refrigerator; before each measurement they were heated for one hour in a thermostat at 37°C and mixed thoroughly. The results are shown in Table 1.

The turbidity of the suspension slowly decreased from the third day after the completion of cultivation. This could be due to spontaneous agglutination in the refrigerator. All subsequent measurements were therefore performed immediately on completion of cultivation (i.e., on the " first " day).

TABLE 1

REPEATED PHOTOMETRY OF NOMINALLY IDENTICAL 14-DAY CULTURE SUSPENSIONS [a]

Culture	Extinction of suspension measured on			
	1st day [b]	2nd day [b]	3rd day [b]	4th day [b]
1	0.79	0.74	0.75	0.77
2	0.87	0.81	0.82	0.82
3	1.10	1.20	0.99	0.92
4	0.88	0.87	0.86	0.85
5	0.87	0.86	0.86	0.82
Average	0.90	0.90	0.86	0.84

[a] Photometer: Pulfrich Elpho, 1 cm cuvette, S57 filter; measured against Dubos cultivation medium with glucose; suspensions diluted 1 : 2.

[b] Counting from the day on which growth was completed.

Accuracy of photometric measurements (Experiment 1)

Measurement, by means of the Pulfrich photometer, of the turbidities of culture diluted in geometrical progression indicated that the system fulfilled the basic prerequisites of photometry—namely, the extinction values were directly proportional to the suspension concentrations (Table 2).

The measurement error was determined as follows: the initial dilution (1: 1) was considered to be unity and in the further dilutions the deviations were calculated. This procedure incorporates the dilution error into the photometric error, but a more accurate procedure was deliberately not adopted. Altogether eight samples were measured and the measurement error was found to satisfy the relation

$$\log E \pm t\sigma_\epsilon \; ; \; \sigma^2_\epsilon = 0.000103$$

This means that, if the value E = 0.5 is obtained, the true value of E lies in the range 0.47—0.53 (P=95%).

Scatter in weighing and photometric methods (Experiment 2)

The eight samples were weighed; some were weighed twice, in which case the mean weight was taken. Photometry was performed twice on each sample. The results are presented in Table 3.

Within each column the values lie within a narrow range. Hence it appears that the cultures were very similar in density and it can be concluded that the cultivation conditions are relatively constant.

TABLE 2
EXTINCTION VALUES OF EIGHT BCG CULTURES

Culture	Extinction at dilution of			
	1 : 1	1 : 2	1 : 4	1 : 8
1	2.0	1.04	0.54	0.27
2	2.05	1.06	0.54	0.30
3	2.1	1.13	0.55	0.28
4	2.09	1.15	0.57	0.31
5	2.15	1.16	0.59	0.34
6	2.12	1.12	0.58	0.28
7	2.3	1.24	0.62	0.34
8	2.2	1.23	0.60	0.33
Average	2.12	1.14	0.57	0.31

The scatter in this experiment is in fact the sum of two factors, one of which is the error inherent in the method (σ_ϵ), whereas the other is the error due to measuring eight independently prepared samples (σ_s). The magnitude of σ_s is probably the same (in logarithmic transformation) for photometry and weighing. Hence, the difference in scatter between the two methods must be due to the respective errors of the procedures.

TABLE 3
ESTIMATION OF BACTERIUM CONTENT OF EIGHT DIFFERENT SAMPLES BY WEIGHING AND BY PHOTOMETRY

Weight (W) (mg/10 ml)	Extinction [a] (E)
6.4	1.24
6.3	1.23
6.0	1.15
5.9	1.16
5.5	1.12
6.5	1.04
6.9	1.06
7.1	1.13
log W (or E) = $\bar{x} \pm \sigma$	
0.7996 ± 0.0324	0.0565 ± 0.0271
σ^2	
0.001322	0.000736

An attempt was made to estimate each component of scatter for the photometer by deriving σ_ϵ by the use of diluted suspensions. The estimate of σ_s thereby obtained made it possible to calculate σ_ϵ for the dry-weight method—namely,

	Experiment 1	Experiment 2
Photometer:	$\sigma_\epsilon^2 = 0.000103$	$\sigma^2 = 0.000736$
		$\sigma_s^2 = \sigma^2 - \sigma_\epsilon^2 = 0.000633$
Dry weight:		$\sigma^2 = 0.001322$
		$\sigma_\epsilon^2 = \sigma^2 - \sigma_s^2 = 0.000689$

Both experiments indicate that, with the present experimental arrangement, photometry is most probably more precise than weighing; however, the significance of the difference in precision cannot be determined.

Comparison of weighing and photometric methods (Experiment 3)

Two cultures of mycobacteria grown in Dubos medium were used in an experiment designed to compare the accuracies of the weighing and photometric methods. The cultures were 14 and 19 days' old. The experiment was performed by different workers and with different apparatus from that used in the two preceding trials. Dry weight was estimated on membrane filters, as before. Ten samples of each culture were used.

Photometry was carried out on 10 samples per culture in two different dilutions, i.e., in 40 samples altogether. Dilution 1 contained 4 ml of culture and 6 ml of medium as diluent; dilution 2 contained 6 ml of culture and 4 ml of medium as diluent. Two photometers were used: Pulfrich (Zeiss-Jena, visual reading) and a photoelectric photometer of Czechoslovak origin. With the Pulfrich apparatus, an S 57 filter and 1 cm cuvettes were used. The measurements were performed independently by two workers, each of whom repeated each measurement three times. The arithmetic mean of all the measurements by both workers was taken as the result for each sample. The measurements with the photoelectric photometer were carried out using 1 cm cuvettes and filter D_2 (yellowish green). With this photometer, each sample was measured only once.

TABLE 4. COMPARISON OF REPEATED WEIGHINGS AND PHOTOMETRIC MEASUREMENTS ON TWO SUSPENSIONS

Weight method		Photometric method							
		Photoelectric photometer				Pulfrich photometer			
Weight (W) (mg/10 ml)	No. of weighings	Dilution 1 [a]		Dilution 2 [b]		Dilution 1 [a]		Dilution 2 [b]	
		Extinction (E)	No. of readings	Extinction (E)	No. of readings	Extinction (E)	No. of readings	Extinction (E)	No. of readings
				Sample A					
4.7	1	0.18	1	0.29	1	0.47	2	0.695	1
5.1	2	0.19	7	0.30	4	0.475	1	0.705	1
5.2	1	0.20	2	0.31	3	0.495	3	0.710	3
5.4	3			0.32	2	0.500	2	0.715	4
5.5	2					0.505	2	0.735	1
5.7	1								
				Sample B					
7.1	1	0.33	5	0.48	1	0.745	4	1 085	1
7.4	1	0.34	5	0.50	8	0.75	4	1.100	3
7.6	1			0.52	1	0.755	1	1.105	3
8.1	1					0.760	1	1.115	2
8.2	1							1.140	1
8.3	3								
8.5	1								
8.9	1								

[a] Dilution 1 = 4 ml culture + 6 ml medium. [b] Dilution 2 = 6 ml culture + 4 ml medium.

The results of the measurements are given in Table 4. The readings are arranged in the columns in increasing order of magnitude; the columns headed " No. of weighings " or " No. of readings " indicate how many times the respective values were obtained. From these values, the selective scatters (σ_ϵ) were calculated. These were compared by means of the F test, as follows—

	σ^2_ϵ		F
Weighing (W)	0.000725	W/E_p	12.7
Photoelectric photometer (E_{el})	0.000117	W/E_{el}	6.2
Pulfrich photometer (E_p)	0.000057	E_{el}/E_p	2.1

Critical value: $F\,0.05\,(9.9) = 3.18$

The precision of the photometric measurements was significantly greater than that of the weighings. The difference between the two photometers was not significant, although the Pulfrich instrument gave the higher precision.

A comparison of the σ^2_ϵ values obtained in this experiment with those estimated in the first two shows that the differences are insignificant—namely

	Expt. 1 & 2	Expt. 3
Photometer (Pulfrich)	0.000103	0.000057
Dry weight	0.000689	0.000725

Hence it is concluded that experiments carried out independently in two different laboratories are fully comparable.

Table 5 gives approximate transformation values (regardless of scatter) for the two methods.

DISCUSSION

The validity of the Lambert-Beer law in a particular instance depends on the optical properties of the liquid under study and the characteristics of the apparatus. Essentially, the liquid is assumed to be a coloured solution the spectral properties of which are not altered by concentration. The photo-

TABLE 5
TRANSFORMATION OF WEIGHT AND EXTINCTION VALUES

Dry weight (W)	Extinction on Pulfrich photometer (E_{570}) [a]
4.5	0.61
5.0	0.67
5.5	0.74
6.0	0.81
6.5	0.88
7.0	0.96
7.5	1.05
8.0	1.10
8.5	1.17

$\log E_{570} = 1.039 \log W - 0.8976$

[a] Extinction of suspension diluted in ratio 6 ml culture to 4 ml medium, measured at 570 mμ in 1-cm cuvette.

meter must be constructed in such a way as to operate with strictly monochromatic light. With bacterial suspensions the situation is somewhat different: strictly monochromatic light is not so essential. Bacteria that are exposed to light act on the one hand as targets that retain light irrespective of wavelength and on the other as point light sources, emitting light in a different direction from that of the original collimator beam. The elimination of these secondary rays depends on the construction of the photometer; wavelength is not a decisive factor. The sensitivity of the photoelectric photometer in measuring bacterial suspensions increases with shortening of the wavelength. Visual photometers, in contrast, are most sensitive in the green region.

The check on the validity of the Lambert-Beer law (Experiment 1) is of importance in that it establishes that the weighing and photometric values are mutually transformable.

The photometric method is suitable only where bacterial growth leads to turbidity, as in the Dubos medium in the present work. Photometry is best performed immediately on completion of cultivation. It is a more precise and more rapid method than weighing.

The lesser precision of the weighing method is explicable in terms of the procedure adopted. First, the weight of the mycobacteria (approx. 5 mg) is too small to be determined accurately on an ordinary analytical balance. Secondly, manipulation of membrane filters, particularly their insertion into the filter head, may easily lead to crumbling of the membrane edge.

RÉSUMÉ

Les auteurs ont comparé la méthode de titrage du contenu en mycobactéries des cultures de vaccin BCG par pesée du vaccin sec et une méthode de titrage photométrique qu'ils ont mise au point. Leurs essais ont été faits sur une culture de BCG poussant de façon homogène sur milieu de Dubos au Tween 80, sans albumine.

Pour la pesée, les bactéries ont été recueillies sur une membrane filtrante. La membrane, après dessication, a été pesée avant et après la filtration. La différence des deux poids a donné le poids de la culture. La méthode photométrique a utilisé un photomètre Pulfrich Elpho et un instrument d'origine tchèque.

Les résultats photométriques ont été les plus précis, à condition d'examiner la culture dans les deux jours qui suivent sa croissance complète; à partir du troisième jour, la densité optique de la suspension décroît lentement, peut-être en raison d'une agglutination spontanée des bactéries.

Le manque de précision de la méthode pondérale est probablement dû au faible poids du dépôt de mycobactéries qui a presque toujours été à la limite de fidélité de la balance analytique et aux difficultés de manipulation des filtres.

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