A RAPID GRAVIMETRIC METHOD OF STANDARDISING VACCINES.

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The method most widely employed in this country for standardising vaccines is that introduced by Sir Almroth Wright in 1902. This observer determined the relative number of microorganisms and corpuscles in a mixture of equal parts emulsion and blood. Since the number of corpuscles present in blood is from $4\frac{1}{2}$ to 5 millions per cubic millimetre, the number of bacteria in a cubic centimetre of the vaccine can be readily deduced.

Wright's original method continues to be employed in the majority of the laboratories of this country, but, as better results were obtained by Leishman (1905) and his colleagues with a modification introduced by W. S. Harrison (1905), this has taken its place in the standardisation of the vaccines prepared for the anti-typhoid inoculation of British soldiers. In the examples given in Wright's original paper, differences as great as 22% exist between the determinations by two different observers of the number of staphylococci in an emulsion. In our hands also, very different results were frequently obtained when the same emulsion was examined two or three times in succession.

Faulty technique alone will not account for the discrepancies, since other observers making consecutive determinations have obtained results very similar to our own. We may quote from a paper by Leishman (1905): "This method (Wright's blood counting method) was accordingly put into practice with various trial samples of

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broth and agar vaccines, but although at times we obtained uniform results which were controlled by a living count made by dilution and plating out on agar, we were unable to obtain consistently satisfactory results, and in the case of broth vaccines errors of from 50 to 100 % in counts of the same film made by different observers were by no means uncommon. All the devices recommended by Wright and many others were employed towards securing a perfect blood film which in all its parts should represent accurately the relation of the number of germs to the number of red corpuscles, but without giving us any greater confidence in the results obtained. The chief factors which appear to interfere with the accuracy of the method are (1) the difficulty in securing a perfect film in which the ratio of germs to cells shall be constant throughout; (2) the clumping or agglutination which frequently occurs, especially in broth cultures, leading to great irregularities in the enumeration of a series of microscopic fields; (3) the part played by the bacteriolytic action of the blood fluids which at times undoubtedly leads to an under-estimation of the number of germs.

"Still at times the results obtained were apparently trustworthy especially with agar emulsions of a certain strain, and in several instances such counts were confirmed by three independent observers."

It is noteworthy that by the employment of Wright's method the strength of a vaccine is, as a rule, under-estimated: this result is probably largely due (as W. S. Harrison (1905) has suggested) to the fact that during the process of fixing and staining a proportion of the bacteria are washed away while the more bulky red cells remain; and also to the possibility of the red cells lying on the top of and thus concealing the bacteria.

Although it will be impossible to obtain accurate dosage of vaccines until the immunising body can be obtained in a more or less pure condition since different strains of bacteria possess different immunising power, the adoption of a gravimetric method of estimating the strength of a vaccine would bring the dosage of bacterio-proteins into line with that of other therapeutic agents.

The gravimetric method is invariably used in standardising Tuberculin and C. J. Martin employed this method in the standardisation of a typhoid vaccine for Leishman (1905) and of a *Micrococcus melitensis* vaccine for Bassett-Smith (1908) in the case of cultures where enumeration was impossible.

In the following paper an attempt has been made to obtain a correlation between dried weight and number of bacteria.

Briefly our procedure was (1) to evaporate to dryness at 40°–50°C. 5 c.c. of an emulsion in distilled water of the growth of various bacteria on agar slopes and to obtain the weight of the dried residue; (2) to enumerate the bacteria in the same emulsion by Wright's method. The evaporation was conducted in a long-necked flask (to avoid loss by splashing) and two Sprengel's water vacuum pumps connected to the flask by two Liebig's condensers produced the necessary aspirating action and vacuum. Twenty minutes sufficed for this process. The flasks when empty and at the end of the process were cautiously heated over a Bunsen flame, placed in a desiccator for five minutes and then weighed after standing ten minutes in the pan of the balance. It was necessary to take the weight after a definite interval as the flasks gained in weight on standing when removed from the desiccator.

The operation was generally repeated two or three times with the same emulsion and as will be seen from our Tables the results were wonderfully constant. By comparing the mean of the different weighings with the mean of the enumeration determinations it was possible to determine the number of bacteria required to weigh one milligramme.

We experimented with the commoner pathogenic germs and in the Tables following are recorded the results obtained. In these Tables are given the weight of the dried bodies of the bacteria present in 5 c.c. of the emulsions employed in the several experiments, whilst in another column are given the numbers of bacteria estimated by Wright's method to be present in 1 c.c. of each of the emulsions. In estimating the number of bacteria we counted on an average 400 red cells in each determination.

By the term error we understand the difference between the maximum and minimum determination and that of the mean expressed as a percentage.

TABLE I.

Staphylococcus aureus.

The growth from eight agar slopes was emulsified in 20 c.c. distilled water.

	Weight in milligr. of residue from 5 c.c. of emulsion	Percentage error	Average error	Millions of cocci per c.c.	Percentage error	Average error
1st emulsion	4·4 4·4 4·1	+2·3 −4·6		2607·6 2524·6 1968·6	+10·1 −16·8	
2nd emulsion	2·6 2·3	±6·1		1127 1128	±0	
3rd emulsion	3·4 3·2 3·0	±6·2	+3·44 −3·38	1230·4 3640·7 2592·5	+47·1 −50	+24 −22·8
4th emulsion	4·8 4·8 5·0	−0 +2·6		3728 2023 1430·3	+55·7 −·40	
5th emulsion	5·6 5·6	±0		4458 3831	±7·5	

Total 53·2 mgrms. for 5 c.c. or 10·64 for 1 c.c. Total 32,387·7 million.

From this Table we see (1) how much more uniform the results are in the gravimetric method, (2) that by taking into consideration all the experiments we can infer that 32,387·7 million Staphylococci weigh 10·64 milligrammes or 3043·9 million dried Staphylococci = 1 milligramme.

TABLE II.

B. typhosus.

The growth from 12 agar slopes was emulsified in 36 c.c. distilled water.

Weight in milligr. of residue from 5 c.c. of the emulsion		Mean	Percentage error	Millions bacilli per c.c.	Mean	Percentage error
1st experiment	2·25			4207·3		
2nd ,,	2·3			4009·9		+ 8·7
3rd ,,	2·4		− 8·1	3395	3870·7	− 12·2
4th ,,	2·75	2·45	+12·2			
5th ,,	2·5					
6th ,,	2·5					

2·45 milligr. in 5 c.c. or ·49 milligr. in 1 c.c. from 3870·7 million bacilli.

This may be stated another way by saying that 7899 million dried typhoid bacilli weigh 1 milligramme.

It is interesting to note that Leishman (1905) and his pupils and C. J. Martin found that the dry residue from 30,390 million typhoid bacilli weighed 3·5 milligrammes or 8683 million bacilli weighed 1 milligramme. We may conclude therefore that there will not be much

error in the assumption that 8000 million dried typhoid bacilli weigh 1 milligramme.

TABLE III.

B. coli communis.

The surface growth from ten agar slopes was emulsified in 20 c.c. distilled water.

Weight in milligr. of dry residue from 5 c.c. emulsion		Percentage error	Average error	Millions of bacilli per c.c.	Percentage error	Average error
1st emulsion	4·9	−2		5192·3	+ 8·8	
	4·9	+4		5426·5	− 13	
	5·2			4336·2		
2nd emulsion	3·8	−7·3	+4·1	5864·6	+ 8·9	+ 8·6
	4·3	+4·8	−4·8	4794·7	− 14	−11·1
	4·2			6081·0		
3rd emulsion	5·4	−5·2		8201·6	+ 8·3	
	5·9	+3·5		9060·7	− 6·4	
	5·8			7818·6		

44·4 milligrammes dry residue from 56776·2 × 5, million bacilli, or 8·88 milligrammes from 56776·2 million.

It is evident that 6393 million *B. coli* weigh 1 milligramme.

TABLE IV.

Meningococcus. (*Weichselbaum's Diplococcus.*)

The growth from seven ascitic-agar tubes was emulsified in 13 c.c. distilled water.

Wt. in milligr. of dry residue from 5 c.c. of emulsion	Percentage error	Millions of cocci per c.c.	Mean	Percentage error
1·3	±7·4	862	796·3	+8·2
1·4		723		−9·2
		804		

Mean 1·35 milligrammes from 5 c.c. 796·3 million per c.c.

From this we learn that 1·35 milligrammes are the weight of (796·3 × 5) million Meningococci or 2948·8 million (3000 million in round numbers) weigh 1 milligramme.

TABLE V.

Micrococcus melitensis.

The growth from six agar tubes was emulsified in 14 c.c. distilled water.

Wt. in milligr. of dry residue from 5 c.c. of emulsion		Percentage error	Average error	Millions of cocci per c.c.		Percentage error	Average error
1st emulsion	2·7	±1·8	±2·1	7980·7		− 6·9	
	2·8			9620	8874·5	+12·1	+6·8
2nd emulsion	1·95	±2·5		8129			−4·2
	1·9			5000		± 1·5	
				4843			

9·35 milligrammes from (26698·2 × 5) million cocci.

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We see then that 14,277 millions of *M. melitensis* weigh 1 milligramme.

It is interesting to note that Bassett-Smith (1908) using the planting out method of enumeration found that 9375 millions weighed 1 milligr. The fact that by the culture method dead cocci could not be enumerated would account for the difference in our results.

TABLE VI.

Pseudo-Gonococcus.

This was a culture of a gram-negative diplococcus obtained from the urethra in a case of Gonorrhoea. It differed from the *Gonococcus* in being more luxuriant in its growth and in staining more uniformly. The growth from six agar-tubes was emulsified in 12 c.c. distilled water.

Wt. in milligr. of dry residue from 5 c.c. of emulsion	Percentage error	Millions of cocci per c.c.	Percentage error
5·2 4·9	±3	3966 5000 {4483 4456	+11·7 −11·3

10·1 milligrammes are present in (8939 × 5) million gonococci.

1 milligramme = 4425 million.

We may take it that 4500 million Gonococci would weigh 1 milligramme.

TABLE VII.

B. pyocyaneus.

The growth from four agar cultures was emulsified in 14 c.c. of distilled water.

Wt. in milligr. of dry residue from 5 c.c. of emulsion	Percentage error	Millions of bacilli per c.c.	Percentage error
4·8 4·4	±4·3	3114 3403 {3258 3058	+6·6 −4·1

9·2 milligrammes from (6316 × 5) million bacilli.

One milligramme of dried bacterial substance is contained in 3432 millions of *B. pyocyaneus.*

TABLE VIII.

B. pneumoniae (Friedlander).

The growth from six agar slopes was emulsified in 15 c.c. distilled water.

Wt. in milligr. of residue from 5 c.c. of emulsion	Percentage error	Millions of bacilli per c.c.	Percentage error
3·1 2·9	±3·3	2677 2549	±2·4

6 milligrammes are contained in (5226 × 5) million bacilli.

4355 million pneumobacilli of Friedlander weigh 1 milligramme.

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Streptococcus.

The growth from six agar cultures of a Streptococcus of the *faecalis* class was smeared on a piece of platinum foil, dried and weighed. The dried residue was then emulsified in 8 c.c. of normal saline solution and the number of cocci per c.c. enumerated. A fairly uniform emulsion was obtained and it was found that the dry residue weighing 1·25 milligrammes was equivalent to 4248 million cocci or 3398 Streptococci weigh 1 milligramme.

In Table IX a summary of our results is given and a comparison is shown of the dosage of the dried bacterial bodies with that of some of the most active drugs of the Pharmacopoeia.

TABLE IX.

Organism	Dose expressed in millions: Minimum	Dose expressed in millions: Maximum	Dose expressed in milligrammes: Minimum	Dose expressed in milligrammes: Maximum
Streptococcus (3400 million = 1 mgrm.) ...	6·8	68	0·002	0·02
Gonococcus (4500 million = 1 mgrm.) ...	45	900	0·01	0·2
Meningococcus (3000 million = 1 mgrm.) ...	300	900	0·1	0·3
M. melitensis (14000 million = 1 mgrm.) ...	700	1400	0·05	0·1
B. coli (6400 million = 1 mgrm.) ...	16	240	0·0025	0·0375
B. typhosus (8000 million = 1 mgrm.) in treatment	100	250	0·0125	0·03125
B. typhosus (8000 million = 1 mgrm.) in prophylaxis	500	1000	0·0625	0·125
B. pyocyaneus (3400 million = 1 mgrm.) ...	34	1020	0·01	0·3
B. pneumoniae Friedlander (4400 million = 1 mgrm.)	44	...	0·01	...
Staphylococcus aureus (3000 million = 1 mgrm.)	150	900	0·05	0·3
B. tuberculosis	...	...	0·00005	0·005

Drugs	Dose expressed in grains: Minimum	Dose expressed in grains: Maximum		
Aconitina	$\frac{1}{640}$	$\frac{1}{400}$	0·1	0·15
Atropina	$\frac{1}{200}$	$\frac{1}{100}$	0·3	0·6
Ergotininae Citras	$\frac{1}{200}$	$\frac{1}{60}$	0·3	1·3
Hyoscinae Hydrobromidum	$\frac{1}{200}$	$\frac{1}{100}$	0·3	0·6
Nitroglycerinum	$\frac{1}{200}$	$\frac{1}{60}$	0·3	1·3
Morphinae Hydrochloridum	$\frac{1}{8}$	$\frac{1}{4}$	8	15
Phosphorus	$\frac{1}{100}$	$\frac{1}{20}$	0·6	3
Acidum Arseniosum	$\frac{1}{60}$	$\frac{1}{15}$	1	4
Strychnina	$\frac{1}{60}$	$\frac{1}{15}$	1	4

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A modification of the method.

The following modification enabled us to rapidly dry and accurately weigh the bacterial bodies.

A rectangular piece of thin platinum foil ($1\frac{1}{2}$ in. × 1 in.) is heated to redness in the flame of a Bunsen burner, is then rolled up and placed in a small sterile glass tube and the combined weight taken. The growth from the surface of an agar culture is removed by a platinum loop and smeared over one surface of the foil which is then replaced in the weighing tube. The latter is now put in the desiccating chamber which consists of a short round bottle with a wide aperture into which a rubber bung is inserted. Penetrating the bung are three glass tubes; two of these are connected to Sprengel pumps by means of Liebig's condensers or simply by glass tubes, whilst the third has a stop-cock and is connected with a little bulb filled with cotton wool which acts as a filter when air is admitted to the desiccator at the end of the experiment. The desiccating bottle is then placed in a water bath or in a hot air incubator at a temperature of 40—54° C.

Complete desiccation occurs in less than 15 minutes; the glass tube and foil are allowed to cool in the desiccator and then weighed. When the weight of the bacterial bodies has been thus obtained the platinum foil is spread out in a sterile porcelain dish, a drop of sterile water is put on the end of a pestle and the dried growth rubbed until a uniform emulsion is obtained, sterile salt solution being gradually added. The same procedure is used as in the preparation of an emulsion of tubercle bacilli but the time required is only 2—5 minutes. The emulsion is placed in a graduated test-tube and to it is added some saline solution which has been used for rinsing out the dish. The vaccine is then diluted to the required extent, sterilised by heating to 53° C.—60° C. Sterility is proved by planting out a loopful and then 0·5 % carbolic acid is added as a preservative.

We have employed the platinum foil method in standardising vaccines made from cultures of *Staphylococcus aureus, Streptococcus faecalis, B. pyocyaneus, B. coli, B. typhosus* etc. and have found that in all cases it is possible to obtain a uniform emulsion from the dry residue and that fifteen minutes in the drying apparatus is sufficient time for complete desiccation.

We may here touch upon the doses that have been used by various investigators in connection with antityphoid inoculations. Wright in his first experiments (1896) employed agar cultures and his first, second

and third doses were $\frac{1}{20}$, $\frac{3}{20}$, $\frac{5}{20}$, of an agar culture. Pfeiffer employed $\frac{1}{10}$ of an agar culture. It is interesting to note that in one of our experiments we found that 0·9 milligramme of dry bacterial bodies was obtained from an agar culture of *B. typhosus* so that an agar slope (a dose of $\frac{1}{16}$ milligramme or 500 million bacteria being taken) would furnish 14 doses.

The British authorities (Wright, Leishman, Harrison etc.) use 500 million dead typhoid bacilli for the first and 1000 million for the second dose; these doses expressed in weight (8000 million bacilli = 1 milligramme) are equivalent to 0·0000625 gramme and 0·000125 gramme of dried bacterial substance.

Pfeiffer and Kolle employed for first, second and third doses, 1, 2 and 3 loopsful respectively. A loopful was taken to be equal to 2 milligrammes of moist growth from an agar slope. If we assume that the growth contained 28 % of dry bacterial solids (a value we have found for moist cultures), the doses given were 0·00056, 0·00112, and 0·00168 grammes of the dry bacterial substance.

The doses given by Basseuge and Rimpau were thirty times smaller than those given by Pfeiffer and Kolle. In round numbers the initial dose of Pfeiffer and Kolle was 9 times greater and that of Basseuge and Rimpau $3\frac{1}{4}$ times less than that of Wright.

In these experiments the dose was given subcutaneously.

Löffler (1904) struck out in a new line in the following respects: (1) he injected the vaccine into the median basilic vein, (2) he dried the bacterial substance in a desiccator, accurately weighed it and sterilised it by heating it in the dry condition to a temperature of 120°—150° C. Knowing that enzymes in the dry condition are very resistant to heat he anticipated that the same would apply to the bacterial antigen and his experiments seemed to confirm this.

Löffler's doses were exceedingly small varying from 0·0156 milligr. to 0·000195 milligr. of the dried bacterial substance.

Friedberger and Moreschi (1905) employed Löffler's method in the immunising of men and animals against *B. typhosus*. They did not, however, heat the dry residue above 120° C. as higher temperatures injured the antigen and prevented a uniform emulsion being obtained.

They used doses similar to those of Löffler, and found that although far smaller doses were used, the antibody building was as marked as when Wright's and Pfeiffer's and Kolle's methods were employed. One great advantage was that there was no local reaction at the site of inoculation whilst the general reaction was the same as in the other methods.

Whether killing the bacteria in a vaccine by desiccation will give better results than the usual method of killing by heat, future work will show. We have observed that the rapid drying in our method, if repeated two or three times in succession (moistening the residue with distilled water each time) is capable of killing typhoid bacilli.

The vaccine as now prepared for the British Army is not heated above 53° C. as it has been found that heating to 60° C. as formerly practised injures the antigen.

Some (*e.g.* Semple and Matson) have employed cultures killed by the addition of 0·5 % carbolic acid and claim that such vaccines are more potent than those sterilised by heat. It is to be noted that there is a difference in the effects whether carbolic acid is added to kill the bacteria or simply as a preservative. Thus 0·5 % carbolic acid diminishes the immunising power of fresh plague cultures whilst the effect is not manifested on dead bacilli (Pick).

It may be that rapid desiccation in some such apparatus as ours may not only assist in the standardisation but also effect the sterilisation of a vaccine with a minimum amount of injury to the antigen.

We may note that Harrison (1907) attempted to kill typhoid bacilli by desiccation but abandoned the method because several days were occupied in the process and the residue would not emulsify. In Harrison's experiments the bacteria were dried in a vacuum desiccator at 37° C. over sulphuric acid. In our method the continuous aspirating action of the Sprengel pumps causes exceedingly rapid desiccation and surmounts the difficulties Harrison encountered. In the following summary we give a concise statement of the results obtained in our investigation.

Summary.

1. A method is described of rapidly evaporating to dryness bacterial emulsions contained in tarred flasks and from the increase of weight determining the strength of the emulsion.

The same apparatus can be used for a variety of purposes, *e.g.* drying serum, estimating solids in water, milk etc.

2. A very rapid method of drying bacteria smeared on a tarred piece of platinum foil, and from the dried residue preparing a vaccine is described.

3. A correlation between the number and weight of bacteria in vaccines made from agar cultures of the *B. typhosus*, *B. coli*, *B. pyocyaneus*, *Meningococcus* (Weichselbaum), *Pseudo-Gonococcus*, *Micrococcus*

melitensis, B. pneumoniae Friedlander, *Staphylococcus aureus* and *Streptococcus* has been established.

4. The advantages of the gravimetric method are:

(*a*) That with a sensitive balance constant results are obtained.

(*b*) It is more rapid, and involves no strain on the eyes.

(*c*) It can be applied to cultures of all bacteria growing on solid media, whether these form uniform emulsions or not, *e.g.* vaccines of *Streptococci, Micrococcus catarrhalis,* Diphtheroid organisms, *B. tuberculosis, Streptothrices* can be accurately standardised. Even small clumps in the vaccine considerably vitiate the results in Wright's method. The chief precaution to take in the gravimetric method is to remove the growth carefully, without breaking the surface of the medium and avoiding the condensation water.

(*d*) It brings into line the dosage of bacterio-proteins with that of other medical remedies.

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