BRITISH MEDICAL JOURNAL

LONDON SATURDAY MAY 10 1947

SOME PROBLEMS IN THE TITRATION OF STREPTOMYCIN

BY

J. R. MAY, M.B., B.Ch. A. E. VOUREKA, M.D.

AND

ALEXANDER FLEMING, F.R.S.

(From the Laboratories of the Inoculation Department, St. Mary's Hospital)

In the titration of an antibiotic substance it has been usual to choose for the test organism a microbe which is very sensitive and which is easy to work with. For streptomycin use has been made of Gram-negative bacilli such as *B. coli* or Friedländer's bacillus, and one strain of Friedländer's bacillus (Klebsiella 41) has been extensively employed in America. In this article we propose to deal with some of the difficulties we have encountered in the titration of streptomycin in solution or in the patient's serum. We might say here that, as we had no official standard solution of streptomycin, we used for these tests a standard solution made up by dissolving in 100 ml. the contents of one bottle of Merck's streptomycin, which was labelled as containing 1 g. of streptomycin hydrochloride.

Influence of the Nature of the Culture Medium

For some time we have been using glucose–phenol-red–serum water for the titration of penicillin in patients' serum. When streptomycin was titrated in this medium we found that we obtained a much higher end-point than when it was titrated in ordinary broth. We therefore made observations on the bacteriostatic power of streptomycin on a number of different organisms in four different media—namely, (*a*) ordinary digest broth; (*b*) peptone water; (*c*) glucose–phenol-red–serum water (this is made by boiling for a few minutes serum, 2 parts; distilled water, 6 parts; 10% glucose solution, 2 parts; and saturated solution of phenol red, sufficient to give a red colour); (*d*) defibrinated human blood, inactivated.

In regard to the first three media serial twofold dilutions of the stock streptomycin were made in the media which had been inoculated with 10 c.mm. per ml. of a 24-hour culture of the test organism. This was done in 0.5 ml. quantities in test-tubes. With broth and peptone water the end-point was the appearance of visible growth after 24 hours. In the serum water there was a colour change from red to yellow, and a precipitation of the medium where the organisms grew and fermented the glucose.

When the test was made in blood the streptomycin was diluted in saline in 25-c.mm. volumes; these were mixed with the same volume of blood which had been suitably infected with the test organism, and incubated in slide cells. As the growth of Friedländer's bacillus in blood is not very obvious 2% glucose was added when testing this organism. When growth then occurred the blood was haemolysed and a good end-point obtained. The results are shown in Table I.

TABLE I.—*Bacteriostatic Power of Streptomycin tested in Different Media*

Test Organism	Lowest Concentration (in Thousands) of Streptomycin Completely Inhibiting Growth in			
	Broth	Peptone Water	Glucose–Phenol-red–Serum Water	Human Blood Inactivated
Haemolytic streptococcus	1/100	No growth	1/2000	1/40
Staphylococcus	1/320	1/1600	1/4000	1/1000
C. diphtheriae	1/600	1/1600	1/4000	1/1000
B. coli (haemolytic)	1/40	1/160	1/1000	1/400
Klebsiella 41	1/320	1/500	1/4000	1/1000*
Friedländer 3	1/1500	1/4000	1/10,000	1/1000*

* A good end-point was obtained only when 2% of glucose was added to the blood.

These results show that there is a great difference in the end-point according to the culture medium used. With one exception the lowest readings were obtained in broth, and invariably the highest readings were those in serum water. It is interesting that the results in human blood are usually considerably higher than in broth.

Influence of Dilution of the Culture Medium

In the first experiments streptomycin solution in distilled water was titrated in broth and in a tenfold dilution of this broth in distilled water. The titrations were done in 0.5 ml. quantities in small test-tubes, and the end-point was the appearance of visible growth. The results are shown

TABLE II.—*Effect of Dilution of the Culture Medium with Water in the Titration of Streptomycin*

Test Organism	Least Concentration Completely Inhibiting Growth of Test Organism in Broth	
	Undiluted	Tenfold Diluted
Staphylococcus	1/300,000	1/60,000,000
Haemolytic streptococcus	1/200,000	1/1,000,000
C. diphtheriae	1/600,000	1/60,000,000
B. coli	1/80,000	1/1,000,000
Klebsiella 41	1/300,000	1/100,000,000

in Table II. In this titration all the constituents of the broth were diluted. Another titration was done in broth and tenfold-diluted broth, to each of which glucose was added to make 2%. This gave the results shown in Table III. In both these experiments there was an enormous difference in the end-point in the diluted medium.

TABLE III.—*Test Organism, Klebsiella 41. Growth in Dilutions of Streptomycin (in thousands)*

	1/100	1/500	1/2500	1/12,500	1/62,500	Control
Broth + glucose ..	0	+	+	+	+	+
Tenfold-diluted broth + glucose	0	0	0	0	+	+

Meantime other experiments had shown that the salt content of the medium had some influence on the end-point, so titrations were made in broth, and in broth diluted ten times with water and with normal saline. The results are shown in Table IV. This showed that the salt content of the medium made a considerable difference.

TABLE IV.—*Test Organism, Klebsiella 41. Growth in Dilutions of Streptomycin (in thousands)*

	1/100	1/500	1/2500	1/12,500	1/62,500	Control
Broth undiluted	0	+	+	+	+	+
„ diluted in saline ..	0	0	0	+	+	+
„ „ in water ..	0	0	0	0	+	+

We made similar observations using serum water as the culture medium. The original serum water (serum 1 part boiled with 3 parts distilled water) was diluted with water and with saline, and to each was added 1% glucose and phenol red. The results appear in Table V.

TABLE V.—*Growth of Klebsiella 41 in Serum Water diluted with Water and with Saline and containing Streptomycin as Under (in thousands)*

	1/1600	1/3200	1/6400	1/12,800	1/25,600	1/51,200	1/100,000	Control
Serum water undiluted ..	0	0	0	+	+	+	+	+
Serum water diluted 10 times in saline 0·85%	0	0	+	+	+	+	+	+
Serum water diluted 10 times in water	0	0	0	0	0	0	+	+

The original serum-water medium has a much lower salt content than normal saline. (The only salt in it is that of the serum, which is diluted four times in water.) When this was diluted with water the titre of the streptomycin was much higher than in the undiluted serum water, but when it was diluted in normal saline it was slightly lower. When the salt content of undiluted serum water was raised to 0.85% it gave exactly the same end-point as did tenfold-diluted serum water with the same salinity.

These experiments confirm the statement which often appears in streptomycin literature, that the salt content is of considerable importance and that saline solutions to some extent inhibit streptomycin.

Effect of the Size of the Inoculum of the Test Organism

Experiments were made to see whether streptomycin behaves like penicillin, which gives the same end-point whatever the inoculum within quite wide limits, or like sulphanilamide, which is very sensitive to changes in the size of the inoculum. The same batch of medium (glucose–phenol-red–serum water) was divided into 4-ml. volumes which were inoculated with 0.1 ml. of serial dilutions of a broth culture of the test organism (Klebsiella 41). A streptomycin solution 1 in 1,000,000 was titrated with each of these media, with the results shown in Table VI.

TABLE VI

Dilution of Broth Culture used as Inoculum	Growth resulting in Concentrations of Streptomycin (in millions)					
	1/2	1/4	1/8	1/16	1/32	Control
1/1	0	0	+	+	+	+
1/10	0	0	±	+	+	+
1/100	0	0	0	+	+	+
1/1000	0	0	0	+	+	+
1/10,000	0	0	0	0	+	+
1/100,000	0	0	0	0	+	+
1/1,000,000	0	0	0	0	+	+

It will be seen that there is a definite change in the end-point as the inoculum gets smaller. The titration is sensitive enough when the inoculum is considerable, and we have found that when very small inocula are used the results may be irregular. We have therefore used as a routine an inoculum of 10 c.mm. of a 24-hour broth culture to each 1 ml. of the culture medium.

Influence of Atmospheric Conditions

Experiments were conducted to see whether anaerobic conditions influenced the end-point. For the most part these were done in a very simple manner by making serial dilutions in 1-ml. quantities in the infected culture medium and then transferring 0.5 ml. to another set of tubes in which the fluid was covered with a layer of melted petroleum jelly. Thus we had one series of tubes fully exposed to the air and another exactly similar set from which air was excluded. It was found that with some test organisms it required, in the absence of air, a higher concentration of streptomycin to inhibit growth completely.

TABLE VII.—*Influence of Anaerobic Conditions on the Titration of Streptomycin. Test Organism, Klebsiella 41. Medium, Glucose–Phenol-red–Serum Water*

	Growths in Dilutions of Streptomycin Solutions				
	1/8	1/16	1/32	1/64	Control
Aerobic	0	0	0	+	+
Anaerobic	0	+	+	+	+

The same result was obtained when the anaerobic tubes were incubated (without the petroleum jelly covering) in an anaerobic jar.

Incubation in Capillary Tubes.—In the past, when titrating penicillin in a patient's serum, we used small volumes and incubated in capillary tubes the serum dilutions with the test streptococcus and human blood. Later we used the same technique but diluted the serum in glucose–phenol-red–serum water. The capillary tubes were sealed in the flame and incubated horizontally stuck in "plasticine" on a microscope slide. When we adopted this last method with streptomycin, using *B. coli* or Friedländer's bacillus as the test organism, the end-point was often obscured by the fact that in several tubes there was only a partial change of colour—one portion of the tube would be yellow and another red. We found also that the end-point was not the same as it was when the fluids were incubated in open tubes. We therefore compared the results obtained by incubating the same fluids in four different ways: (*a*) in 0.5-ml. quantities in open test-tubes; (*b*) in capillary tubes (about 1 in. (2.5 cm.) column of fluid) (b1) unsealed and incubated horizontally, (b2) sealed and incubated horizontally, (b3) sealed and incubated vertically.

TABLE VIII

Method of Incubation	Dilution of Streptomycin Solution				
	1/8	1/16	1/32	1/64	Control
Test tubes, open	0	0	0	+	+
Capillaries, open, horizontal	0	0	0	+	+
„ closed, horizontal	0	±	+	+	+
„ „ vertical	±	±	+	+	+

The results with open test-tubes and open capillaries were identical, but when the capillaries were sealed (in the flame) it required more streptomycin to inhibit growth and still more when they were incubated vertically. It seems possible that this may, in part at least, be due to the diminished access of air in the closed tube. This result was obtained with *B. coli* and Friedländer's bacillus and staphylococcus, but when streptococcus was used as the test organism the end-point was the same whether the capillaries were open or closed, and whether they were incubated horizontal or upright.

The fact that open capillaries gave what is apparently the true reading is fortunate in that it saves the time which would be occupied in sealing them. The capillaries used were about 4 in. (10 cm.) long and approximately 1 mm. in diameter. When stuck horizontally in plasticine the fluid (unless there is rough handling) remains in place near the middle.

Sensitivity of Different Bacteria to Streptomycin

Many tables have been published giving the amount of streptomycin necessary to inhibit the growth of different bacteria. In a consideration of these figures we must take into account the medium in which the test was made, for, as we have seen, the nature of the culture medium makes a vast difference to the result.

TABLE IX.—*Sensitivity of Bacteria to Streptomycin in Broth and in Glucose-Phenol-red-Serum Water*

	No. of Strain	Lowest Concentration Completely Inhibiting Growth			
		Broth		Serum Water	
		Lowest	Highest	Lowest	Highest
Friedländer's bacillus	5	1/30,000	1/1,500,000	1/2,000,000	1/12,000,000
B. coli	18	1/40,000	1/160,000	1/500,000	1/2,000,000
Ps. pyocyanea	5	1/20,000	1/40,000	1/1,000,000	1/4,000,000
B. proteus	6	1/10,000	1/20,000	1/500,000	1/2,000,000
B. typhosus	8	1/20,000	1/80,000	1/500,000	1/4,000,000
B. paratyphosus A	4	1/20,000	1/40,000	1/1,000,000	1/2,000,000
B. paratyphosus B	5	1/20,000	1/40,000	1/500,000	1/1,000,000
B. paratyphosus C	1	1/20,000		1/1,000,000	
Other Salmonella	6	1/20,000	1/160,000	1/500,000	1/2,000,000
B. dysenteriae	4	1/160,000	1/160,000	1/1,000,000	1/1,000,000
Haemolytic streptococcus	1	1/160,000		1/1,000,000	
Streptococcus viridans	7	1/50,000	1/400,000	1/1,000,000	1/6,000,000
Staphylococci	2	1/320,000	1/320,000	1/4,000,000	1/4,000,000
H. influenzae (Fildes extract added)	4	1/100,000	1/100,000	1/600,000	1/600,000

In Table IX we set forth the results obtained with a large number of organisms tested in our ordinary nutrient broth and in glucose–serum water. When figures are given as to the effect of streptomycin on bacteria it is quite obvious that the culture medium should be described, and it is also very desirable that a standard organism should be included in the test—such as the staphylococcus, which has been universally used for penicillin titration, or Friedländer's bacillus (Klebsiella 41).

Distribution of Streptomycin in Blood.—Experiments were made to determine the relative amount of streptomycin in the corpuscles and the serum. A citation of one of these will serve.

Streptomycin 1/500,000 was added to serum. This was divided into two parts. One of these was mixed with an equal volume of packed cells which had not previously been in contact with streptomycin. At intervals a sample of the mixture was centrifuged and the streptomycin content of the serum was compared with that of the control serum, to which no cells had been added. After one hour the bulk of the streptomycin remained in the serum and only a relatively small amount had passed into the corpuscles, but after six hours the streptomycin was equally distributed between the serum and the corpuscles.

Testing of a Patient's Serum for Streptomycin.—As with penicillin it is desirable, in order to avoid undue bleeding of the patient, to use a micro-method, and the methods employed for penicillin are applicable to streptomycin, with a possible change of the test organism. Normal sera containing no streptomycin were tested by making dilutions in glucose–phenol-red–serum water infected with *B. coli* or Friedländer's bacillus, and it was found that growth was inhibited in the higher concentrations of serum. Sometimes it was only a 1 in 2 dilution which inhibited, but sometimes as much as 1/32 dilution showed no growth. When the serum was inactivated this bacteriostatic action disappeared. It follows from this that if this organism is used the serum must be inactivated, otherwise it will be impossible to detect small amounts of streptomycin in the serum.

Use of Liquoid for Inactivation.—Inactivation of the serum by heat meant another operation, and we thought that by adding liquoid to the culture medium we might destroy the antibacterial power of the serum. Liquoid in a concentration of 1 in 2,000 did not interfere with the growth of any of the test organisms, and it completely destroyed the antibacterial power of the serum. We thought we had solved this problem until we discovered that liquoid interfered in some way with the action of streptomycin, as is shown in the following experiment (Table X). Different concentrations of liquoid were made in serum-water medium, and solutions of 1 in 200,000 streptomycin in the same concentration of liquoid were titrated in each of these media.

TABLE X

Concentration of Liquoid in Medium	Growth of Staphylococcus in Concentrations of Streptomycin (in thousands)						
	1/400	1/800	1/1600	1/3200	1/6400	1/12,800	Control
1/500	0	+	+	+	+	+	+
1/1000	0	0	±	+	+	+	+
1/2000	0	0	0	±	+	+	+
Control, no liquoid	0	0	0	0	±	+	+

This interference of liquoid with streptomycin made it an unsuitable inactivating agent, and when using a test organism which is inhibited by human serum such serum has to be inactivated by heat before its streptomycin content is estimated. It was found that inactivation of the serum by heat (half an hour at 56° C.) did not destroy the streptomycin.

The method of titrating streptomycin in blood serum which we have finally adopted is as follows:

1. *Culture Medium.*—Serum 1 part, distilled water 4 parts, boiled or steamed with 1% glucose and sufficient of a saturated watery solution of phenol red to give a very definite red colour. For use 1 ml. of this medium is inoculated with 10 ml. of a 24-hour broth culture of the test organism.

2. *Test Organism.*—The Friedländer's bacillus (Klebsiella 41) which has been in use in America is very suitable, but if this is employed the patient's serum must be inactivated by heat before the test. Staphylococcus may be used (choosing a strain which is not inhibited by human serum). It is almost as sensitive as Klebsiella 41 and there is no need to inactivate the serum. If the patient has had penicillin, staphylococcus cannot be used unless penicillinase is added to the medium.

3. Serial dilutions of the serum to be tested are made in normal saline solution, and to each of these dilutions is added an equal volume of the infected medium. We have preferred to use .025-ml. volumes and carry out the incubation in capillary tubes *open at both ends* and stuck flat on plasticine on a microscope slide, but the test can be done in exactly the same way using larger volumes and incubating the mixtures in small test-tubes. When serum water is used as the medium for titration and Klebsiella 41 or staphylococcus as the test organism the end-point comes at a dilution of 1 in 4–6 million. In blood it requires about 1 in 1 million concentration of streptomycin to inhibit growth completely, so that this method will indicate a smaller amount of streptomycin than the therapeutic level, whereas if broth is used this would not be so. Serum water is also more suitable for the test as it is an unbuffered medium and the end-point is more easily read than it is in broth.

4. *Control.*—As the final end-point of the titrations depends on so many factors it is necessary to make a control titration of a known concentration of streptomycin (1 in 1,000,000) in human serum. A comparison of the end-point obtained with this and with the patient's serum will give an absolute measurement of the streptomycin content in the latter.

The accompanying Chart illustrates the result obtained by titrations of patients' serum following intramuscular

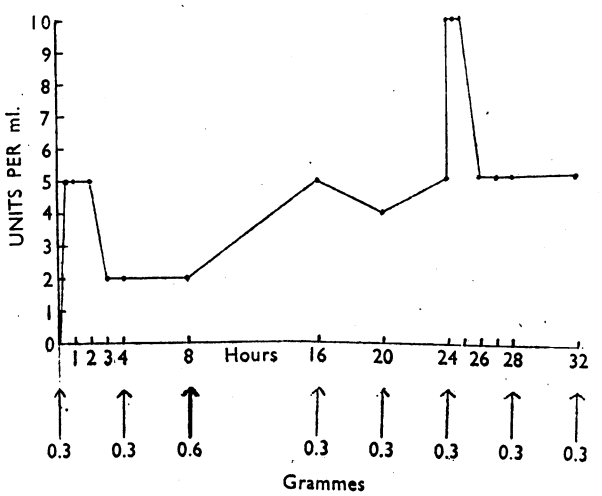

Chart showing the streptomycin content of a patient's serum after intramuscular injections of 0.33 g. of the drug. The arrows represent time of injections.

injections of 0.33 g. of streptomycin. Injections were given every four hours except that at bedtime that amount was doubled (indicated on the chart by a thick arrow), and eight hours elapsed before the next injection. The blood was tested just before each injection and at shorter intervals after the first and the sixth injections. It will be seen that at the end of the first injection interval there were still 2 units per ml. in the serum, but after three injections the residual amount was maintained at 4 to 5 units.

ADDENDUM

Error in making Serial Dilutions with a Capillary Pipette

The usual method is to lay out on a paraffined slide a number of equal volumes from a graduated capillary pipette. None of these is the whole volume represented by the graduation; all of them are that volume less the wash of fluid which wets the inside of the pipette. This varies with the bore of the pipette and with the rate of expulsion, but Fleming (1924) estimated that with ordinary capillary pipettes and at the normal rate of working the wash amounted to about 3%.

The fluid to be diluted is taken up to the graduation mark in the pipette, expelled into the first drop of the diluent, and then mixed by sucking up and expelling the fluid several times. This means that the whole of the fluid to be diluted is mixed with the same volume of the diluent minus the "wash." Then one volume of this mixture is taken with the same pipette and mixed in the same way with the second drop of diluent. But the pipette in making the first mixture has been wetted up to the second graduation mark, so that in making the second dilution the whole volume plus an additional wash is mixed with the volume minus a wash. This is an error accumulating with each of the serial dilutions, and while it is small with a short series it becomes serious when ten or more serial dilutions are made in this way. The extent of the error may be seen in the following experiment.

Dilutions of streptomycin were made accurately 1/10,000, 1/50,000, 1/1,000,000, and 1,/5,000,000. These were titrated by twofold dilutions in glucose–phenol-red–serum water infected with Friedländer 3. With 1/10,000 strength 14 serial dilutions were made before an end-point was reached. Complete inhibition of growth occurred in what purported to be 1 in 80,000,000 and growth occurred in 1 in 160,000,000. With the 1 in 50,000 strength 11 serial dilutions gave an end-point of inhibition in 1/50,000,000 and growth in 1/100,000,000. With the 1/1,000,000 strength 5 dilutions showed inhibition in 1/16,000,000 and growth in 1/32,000,000. With the 1 in 5,000,000 strength 2 dilutions showed inhibition in 1/10,000,000 and growth in 1/20,000,000. A control series done by a method which eliminates the wash gave the same result as the last.

It is quite evident, therefore, that a *long* series of twofold dilutions made in the usual way by a method in which there is the cumulative error of the wash may give results which are wide of the mark. It is suggested that the series of dilutions made in this way should not exceed 5 or 6.

Fleming (1924) has suggested a method whereby dilutions can be made with capillary pipettes, which avoids the error of the wash and can be adapted to the titration of streptomycin or other substance.

Summary

In the titration of streptomycin the end-point depends on (1) culture medium, (2) atmospheric conditions, (3) nature of the test organism, and (4) size of inoculum of the test organism.

Results are given for many organisms in broth and serum water.

A method of titration of streptomycin in patients' serum is described.

Attention is drawn to an error inherent in the method of serial dilutions by capillary tubes.

REFERENCE

Fleming, A. (1924). *Brit. J. exp. Path.*, **5**, 148.

The Royal Sanitary Institute will hold its 1947 health congress at Torquay, from June 2 to 6, at the invitation of the Corporation. The Earl Fortescue, Lord Lieutenant of the County of Devon, will be President of the Congress. The following is a selection from the provisional programme: June 2, 3 p.m., official welcome by the Mayor of Torquay and inaugural address by the president of the congress. June 3, 10 a.m., Section of Preventive Medicine, discussions on "The Scope of Public Health after 1948" and "The Present Position and Prospects in Whooping-cough Immunization"; Section of Engineering and Architecture, discussions on "The Design and Location of Health Centres" and "Engineering Standards in Relation to Health"; Sanitary Inspectors conference, discussions on "The Social Aspect of the Housing Problem" and "Milk: A Food for Thought." June 4, 10 a.m., Medical Officers of Health conference, discussion on "The National Health Service Act and the Public Health Service." Health Visitors conference, discussion on "The Expanding Duties of the Family Health Team." June 5, 10 a.m., Section of Maternal and Child Health, discussion on "Infantile Mortality: the Clinical Aspects"; Section of Tropical Hygiene, discussion on "The Practical Application of Recent Advances in Tropical Medicine and Hygiene to Rural Tropical Areas, (*a*) Anopheles (Malaria) Eradication in Karpas, Cyprus, 1946, and (*b*) Native Welfare in its Wider Aspects, including Nutrition and Housing." June 6, 10 a.m., Section of Maternal and Child Health, discussion on "Infantile Mortality: The Preventive Aspects"; Section of Veterinary Hygiene, discussion on "The Importance of Cowshed Hygiene in the Transmission of Milk-borne Diseases"; Section of Food and Nutrition, in conjunction with the Food Group of the Society of Chemical Industry, discussion on "The Microbiological Aspects of Food Quality."