V. OBSERVATIONS ON THE ACCURACY OF DIFFERENT METHODS OF MEASURING SMALL VOLUMES OF FLUID.

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(Received February 13th, 1919.)

EVERY serologist has his own favourite method of measuring the small volumes of different fluids which he needs in performing complement fixation reactions or in making a series of serum dilutions for agglutination. He usually regards the method to which he is accustomed as the best and may resent any imputation on its accuracy, but few serologists take the trouble to check the method they employ by careful control observations. There is no convenient method for determining the percentage of serum in a dilution sufficiently closely, but there are plenty of chemical substances which can be substituted for serum. One cannot, indeed, carry out volumetric analyses on the small volumes involved in Wright's method of preparing serum dilutions in a capillary pipette, but one can use larger volumes in the same way and analyse the results satisfactorily and can very safely reason that errors detected with the larger volumes are likely to be exaggerated with the smaller.

I have lately had occasion to enquire into possible errors in my own serological technique and I have carried out a certain number of observations, as carefully as I was able, on the lines suggested above. Although there is nothing astonishing in the results it may be of service to put them on record. It has been a gain to me to ascertain the degree of my own error and it would probably be a useful exercise for any serologist to check his methods in similar fashion.

The fluids chosen for measurement were (1) a saturated solution of iodine in saturated potassium iodide solution, and (2) a not quite saturated solution of calcium chloride in water. The latter was on the whole the more satisfactory, being cleaner to work with. The dilutions were titrated in the ordinary way, the iodine with $N/10$ and $N/100$ thiosulphate and a starch indicator, and the calcium chloride with $N/10$ and $N/100$ silver nitrate and a potassium chromate indicator. My thanks are due to Dr W. H. Hurtley, Lecturer on Chemistry at St Bartholomew's Hospital, for kind advice and help in the matter.

There are two ways of measuring fluids, apart from weighing, which is out of the question for serological work. One is measurement by pipette, and the

other measurement by drops, and I was anxious to compare the accuracy of these two methods.

In measuring by pipette the serologist is at a disadvantage as compared with the chemist. Volumetric analysis has been developed into a fine art by generations of chemists and the degree of accuracy obtainable is very high. The chemist has his standard burettes and pipettes and has been trained how to use them. Unless the serologist has been trained as a chemist he is apt to be less scrupulous in the use of the instruments at his command, and he lies under the further disadvantage that he commonly has to measure very small quantities of fluid by means of instruments much smaller and more liable to error than those used by the chemist. The first problem that arises is therefore the degree of error likely to be present in careful pipette measurements of small volumes of fluid, ranging from 1 cc. to 0.01 cc., such as the serologist often has to employ.

THE RANGE OF ERROR IN SMALL PIPETTE MEASUREMENTS.

Those who work with graduated 1 cc. and 0.1 cc. pipettes usually employ as motive force either a rubber teat or a mouthpiece connected with the pipette by a rubber tube. Some such method is necessary with a 0.1 cc. instrument, though with a 1 cc. pipette one can work by gravity, controlling the flow with the finger only.

To check the accuracy of delivery of small volumes from such pipettes the following procedure was adopted. A concentrated solution of calcium chloride was carefully titrated against $N/10$ AgNO_3 : 1 cc. of the CaCl_2 solution was found equivalent to 61.4 cc. $N/10$ AgNO_3 ; the measurement of the 1 cc. was performed with great care by Dr Hurtley. Two Lautenschläger pipettes were washed out with strong sulphuric acid and then repeatedly with distilled water: one was a 1 cc. pipette graduated in tenths and hundredths, the other a 0.1 cc. pipette graduated in hundredths and two-hundredths. A well-fitting rubber teat was attached to each. I then attempted to deliver into evaporating dishes amounts of the calcium chloride solution varying from 1 cc. to 0.01 cc., and titrated them against AgNO_3 , using a decinormal solution for the larger and a centinormal one for the smaller amounts. Even 0.01 cc. of the calcium chloride solution required 6 cc. of centinormal AgNO_3 , so that the titration could be carried out with considerable accuracy. At the same time my colleague, Dr R. G. Canti, who was provided with a similar pair of Lautenschläger pipettes, which he was accustomed to use with a mouthpiece and rubber tube, performed similar measurements. There was thus an opportunity for comparing the teat with the mouthpiece as a motive force.

The results of this experiment are shown in the following table. In the second column is shown the expected value of each amount, assuming the correctness of the preliminary titration, in the third and fifth columns the value actually found, and in the fourth and sixth the percentage by which the value found differed from what it ought to have been.

Table I. *Errors in measurements with small graduated pipettes.*

Measurements with 1.0 cc. pipette.					
Amount measured	Correct value in cc. $N/10$ AgNO_3	F. W. A. using test		R. G. C. using mouthpiece	
		Value found	% deviation	Value found	% deviation
1.0 cc.	61.4	60.8	- 0.98	60.0	- 2.28
1.0	61.4	61.9	+ 0.81	62.0	+ 0.97
1.0	61.4	61.1	- 0.49	—	—
0.5	30.7	30.5	- 0.66	—	—
0.1	6.14	5.7	- 7.17	6.0	- 2.28
0.1	6.14	5.75	- 6.36	—	—
Measurements with 0.1 cc. pipette.					
0.1 cc.	6.14	6.0	- 2.28	6.15	+ 0.16
0.1	6.14	—	—	6.5	+ 5.86
0.05	3.07	2.95	- 3.91	3.015	- 1.79
0.01	0.614	0.605	- 1.47	0.68	+ 10.74
0.01	0.614	0.75	+ 22.15	0.775	+ 26.22
0.01	0.614	0.635	+ 3.42	0.74	+ 20.52

The chief interest of these figures is that they represent the actual results obtained by two ordinary workers, reasonably practised in pipette measurements, and done with extra care because there was a certain emulation as to who could produce the best results. Doubtless there are many workers who could do better: we only regard ourselves as representing the average¹.

It is evidently possible to measure 1 cc. or 0.5 cc. with reasonable accuracy: my own four measurements of this order were all within 1 % of the correct amount. Attempts to deliver 0.1 cc. from a 1 cc. pipette were less successful. This quantity can be more accurately delivered from a 0.1 cc. instrument, and one of Dr. Canti's efforts was almost exact; but he failed to repeat it. The margin of error is clearly greater with the smaller instrument, and when it comes to delivering 0.01 cc. the error is apt to become a serious one. The grosser errors were always in the direction of delivering too much, probably owing to transfer of fluid on the exterior of the nozzle. It is clearly unwise for the average worker to rely on the measurement of one hundredth of a cc. from a pipette with any accuracy.

Two further points came out in the course of this experiment, though they are not indicated in the table. A slightly larger volume is delivered when the pipette is held vertical and emptied slowly. In delivering one-tenth of the total graduated length, more accuracy is attained by measuring one of the middle tenths than the terminal one.

¹ I communicated my results to Dr Carl Browning of the Middlesex Hospital, who thereupon undertook some similar observations—using a narrow 0.1 cc. pipette, provided with a rubber tube and mouthpiece. The fluid used was hydrochloric acid, and the measured volumes were titrated against $N/300$ sodium hydrate. Twenty successive measurements of 0.01 cc. were made, and of these sixteen differed from the mean by less than 2 %, while the extreme variations from the mean were less than 5 %. Eighteen measurements of 0.03 cc. were also made, and fifteen of these were within 1.3 % of the mean, while the extreme variations from the mean were 2.9 %. These efforts are clearly better than those of Dr. Canti and myself.

ERRORS INCIDENT TO THE PREPARATION OF A SERIES OF DOUBLING
DILUTIONS.

Inasmuch as this is one of the commonest procedures in agglutination work, considerable interest attaches to the accuracy of the different methods by which it can be carried out, and especially to a comparison of the pipette and drop methods. I therefore performed a number of experiments with this object in view.

In order to have an idea of the standard of accuracy which might be attainable I invited Dr Hurtley to prepare me a progressive series of doubling dilutions of the same calcium chloride solution as was used in other experiments. The solution was at this time slightly stronger: (this was the first time it had been used); the preliminary titration showed that 1 cc. = 61.6 $N/10$ AgNO_3 . Dr Hurtley prepared the dilutions with great care, employing all the precautions incident to volumetric analysis, and even washing out and drying the pipette between successive measurements. The volume measured at each step was 2.5 cc. Five dilutions were prepared, from 1 in 2 to 1 in 32, each being made from the one preceding it, so that any error might be cumulative. The results are shown in Table II.

Table II. *Deviations from accuracy in a series of dilutions prepared by a chemist.*

Primary solution; 1 cc. = 61.6 $N/10$ AgNO_3 .			
Dilution	Correct figure	Result on titration	% deviation from correct figure
1 in 2	30.8	30.65	-0.5
1 in 4	15.4	15.45	+0.3
1 in 8	7.7	7.85	+1.9
1 in 16	3.85	3.80	-1.3
1 in 32	1.92	2.00	+4.1

The results seen in this table show that a trained chemist, using adequate methods, can prepare a series of desired dilutions with a high degree of accuracy. The slight apparent errors are now +, now -, which suggests that they may depend upon errors in titration rather than in the preparation of the dilutions.

But no serologist can work in this laborious way. He usually employs his pipette in quite a different manner. It is usually not a "delivery" pipette, but one graduated to the point and he blows out the contents as completely as he can. This process inevitably leads to error unless the instrument is dried out at each operation, and what is more, in preparing a series of doubling dilutions in the ordinary way, the error is cumulative. In order to get some idea of how great it may become, I made the two following experiments, using a delivery pipette in the manner of a serologist.

I took an ordinary 4 cc. pipette and measured into each of eight test tubes 4 cc. of distilled water, as carefully as possible but expelling the whole amount of fluid by blowing. The pipette was then dried out, and 4 cc. of strong calcium

chloride solution (1 cc. = 61.5 cc. $N/10$ $AgNO_3$) added to the first tube. The mixture was taken up into the pipette and expelled six times, to ensure thorough mixing and to wash out the pipette, and then, without any other washing or drying, 4 cc. of the mixture were transferred to tube 2 and the process repeated, and so on to the eighth tube, the pipette being at each stage washed out six times with the mixture just made. The results on titration were as follows:

Table III. *Showing cumulative error from incorrect use of pipette.*

Primary solution; 1 cc. = 61.5 cc. $N/10$ $AgNO_3$.			
Dilution	Correct figure	Result on titration	% deviation from correct figure
1 in 2	30.75	31.0	+ 0.81
1 in 4	15.375	15.825	+ 2.92
1 in 8	7.687	8.05	+ 4.72
1 in 16	3.843	4.0	+ 4.08
1 in 32	1.921	2.06	+ 7.23
1 in 64	0.960	1.04	+ 8.33
1 in 128	0.48	0.535	+ 11.45
1 in 256	0.24	0.275	+ 14.58

This series illustrates very well the progressive nature of the error in a short series of dilutions when the pipette is wrongly used. If it can amount to 14 % using so large a pipette as one of 4 cc., how much greater must it be with a capillary pipette in which the surface wetted bears a far larger proportion to the volume of fluid measured.

A method of preparing serum dilutions sometimes employed is as follows: Supposing one has three rows of tubes to fill, as in an ordinary T.A.B. agglutination, a tenfold dilution of the serum is prepared, and one volume placed in each of the first three tubes. Three volumes of the tenfold diluted serum are then put in a watch-glass and three volumes of saline added and mixed. A volume of this mixture is placed in each of the second three tubes, leaving three in the watch-glass, to which a further three volumes of saline are added for the third dilution and so on. The pipette is thus alternately wetted with pure saline and with the dilution at which one has arrived. If, on reaching the end, the volume remaining in the watch-glass is measured, it is never three volumes, but always less, by a half or a third of a volume.

I have tested the degree of inaccuracy of this obviously inaccurate method in the following manner. I used a home-made pipette of narrow glass tubing, holding about 2 cc. to the top mark: it was actuated by a rubber bulb. The solution employed was a strong one of iodine in potassium iodide. I put two pipettesful of water into a glass dish, washed out the pipette several times with iodine solution, and then added two pipettesful of iodine solution to the dish and mixed well. Two pipettesful were transferred to tube 1, and then two more of water added to the dish and mixed. Eight tubes were thus filled in succession. The results on titration with sodium thiosulphate are seen in Table IV.

Table IV. *Showing cumulative error from erroneous technique.*Primary solution; 1 cc. = 61·14 cc. *N*/10 thiosulphate.

Dilution	Correct figure	Result on titration	% deviation from correct figure
1 in 2	30·57	30·7	+ 0·42
1 in 4	15·28	15·1	– 1·8
1 in 8	7·64	7·45	– 3·8
1 in 16	3·82	3·49	– 8·7
1 in 32	1·91	1·63	– 14·7
1 in 64	0·955	0·795	– 16·8
1 in 128	0·477	0·36	– 24·6
1 in 256	0·238	0·175	– 26·5

Whereas in the preceding experiment the error was a progressive one on the + side, this one shows a similar and worse error on the – side. The direction of the error depends on the precise departure from the proper use of the pipette. These are not the only experiments of the sort which I have made, but all have shown the same sort of thing. These are selected and set out here in detail to illustrate the fact that an error which appears negligible in the individual measurement becomes a serious one when it is cumulative. I know of no way in which a serologist is likely to use a pipette which will avoid such error: this is not said in blame, for he has not the time to employ the technique of the trained chemist. But he ought to know what his error is liable to be.

THE DROP METHOD OF MEASURING.

All the sources of error which attend measurement by pipette are entirely avoided in measurement by drops, and in their stead we encounter the new set of difficulties which attends the delivery of equal drops. These however are more easily overcome than in the case of the pipette. Donald has shown us a simple technique by which, with a little practice, it is easy to deliver substantially equal drops of different fluids. [1915, 1916.]

The three important factors governing the size of a drop issuing from a vertically held pipette are (1) the external diameter of the nozzle, (2) the surface tension of the fluid, and (3) the rate of dropping. By the aid of a wire and drill gauge, such as can be bought at any good tool shop, pipettes of constant size can be made as they are needed. The variation in surface tension of different fluids, such as normal saline, fresh human serum, or phenolated rabbit serum, can be compensated by determining which holes in the gauge will furnish pipettes delivering sensibly equal drops of these fluids: once determined this has not to be done again. With a little practice any one can keep his drop rate reasonably constant at about one per second. All this can be read in detail in Donald's published papers.

There are two ways in which one may employ the method in measuring small volumes of fluids of different surface tension. With a single pipette one can determine the relative size of the drops furnished by each fluid, keeping the conditions constant—*e.g.* by ascertaining how many drops of each go to

make up 1 cc. It is then easy to calculate how many drops of each must be mixed to form any desired dilution. The objection to this way of working lies in the impossibility of measuring fractions of a drop. The second plan, that of calibrating two pipettes to deliver equal drops, is therefore much to be preferred.

In endeavouring to test the accuracy of the drop method, I prepared a series of eight doubling dilutions of strong calcium chloride solution. The drops of this solution and of distilled water were found to be so nearly equal in size (256 to 245) that I did not attempt to make two pipettes to deliver equal drops, but adopted the first of the two plans just mentioned; I used one pipette, calculating the numbers of drops required and neglecting fractions of a drop. I did not therefore employ the method in the best way; nevertheless, as will be seen from Table V, the results were surprisingly good.

Table V. *Showing smallness of error with drop method.*

Primary solution of CaCl_2 ; 1 cc. = 61.5 cc. $N/10$ AgNO_3 .

Dilution	Correct figure	Result on titration	% deviation from correct figure
1 in 2	30.75	31.25	+ 1.6
1 in 4	15.375	14.8	- 3.7
1 in 8	7.687	7.65	- 0.5
1 in 16	3.84	3.85	+ 0.3
1 in 32	1.92	1.95	+ 1.5
1 in 64	0.96	0.945	- 1.6
1 in 128	0.48	0.49	+ 2.1
1 in 256	0.24	0.265	+ 10.4

It will be seen that the errors here are, with the exception of the eighth dilution, of the same small order as in the series of dilutions prepared by Dr Hurtley by "chemical" use of the pipette (see Table II). The result is very much better than anything I myself could achieve with a pipette. On the first two occasions on which I tried to make this series of dilutions the results had been wide of the mark, owing to miscalculation of the relative size of the drops of the two fluids. After Mr Donald had been so kind as to visit my laboratory and to point out certain mistakes in technique, the thing became easy, and the above figures represent my third attempt. The dropping was done at constant rate from a burette, provided with a Marriott's tube to keep the pressure even, and not by hand.

The advantages claimed by Donald for his drop technique appear to me to be justified. Anyone can make and calibrate his own pipettes, easily and cheaply as they are required. There is no cumulative error in preparing an ascending series of dilutions. The drop method can be used with the same accuracy in measuring very small volumes as larger ones, whereas it is with the small measurements frequently required by the serologist that the pipette method shows up to such disadvantage. Lastly, provided that the conditions for its use are duly observed, the drop method seems intrinsically more accurate than measurement by pipette.

CONCLUSIONS.

The figures which have been given above are doubtless less exact than could have been obtained by weighing. Any errors which may have been incident to the process of volumetric analysis are of course superadded to those depending on faulty technique in the preparation of the series of dilutions. The errors of titration are probably, however, less than 1 % and I believe that the results obtained are of sufficient accuracy to allow of a fair estimate of the degrees of error which may attend the measurement of small volumes of fluid in serological work.

The chief conclusions which appear to follow from these few observations are as follows:

(1) Volumes of 1 cc. and 0.5 cc. can be measured by pipette with reasonable accuracy. The error attendant on the attempt to deliver 0.1 cc. may amount to 5 %, even when a 0.1 cc. pipette is employed. The delivery of 0.01 cc. from a pipette may be exceedingly inaccurate.

(2) The only way in which really accurate results can be obtained with a pipette in preparing an ascending series of dilutions, is to use it as a delivery pipette and to wash and dry it out between successive measurements. The alternative method, that of using it as a delivery pipette, mixing the dilution by shaking, and then washing out two or three times with the mixture, rejecting the washings, is one hardly adapted for serological work. Unless such precautions are taken the small error introduced, perhaps only one of 0.5 % in a single measurement, though probably much more with a capillary pipette, becomes magnified into one of 10 % or more at the eighth dilution.

(3) The drop method is greatly to be preferred, for serological work, to the pipette method, provided it is properly carried out with calibrated pipettes.

It may be urged that in a process such as the agglutination test, beset with liabilities to inaccuracy, an error of 10 % or so in the serum dilutions is not of much moment. For rough work perhaps it is not, but for accurate work it is. Improvement in agglutination technique can only be brought about by endeavouring to correct the individual sources of error which attend it. This is equally true of such methods as are involved in the technique of the Wassermann reaction. My excuse for publishing these few observations is that I have learned a good deal from them myself, and that it is possible that others may find them not without interest.

REFERENCES.

- Donald (1915). *Lancet*, ii, 1243.
— (1916). *Lancet*, ii, 423.