J. Hyg., Camb. (1965), **63**, 369
With 2 plates
Printed in Great Britain

Description and analysis of a simple micro-titration immune cytolytic test

By R. L. SPOONER
Department of Pathology, University of Cambridge
and F. W. BOWDEN and R. G. CARPENTER
Department of Human Ecology, University of Cambridge

(*Received* 3 *December* 1964)

INTRODUCTION

Immune cytolytic tests so far described (Gorer & O'Gorman, 1956; Hanks & Wallace, 1958; Reif & Norris, 1960; Boyse, Old & Thomas, 1962), all involve separate diluting and reading stages in their performance. The technique of Boyse *et al.* (1962) still requires master dilutions of antiserum, although incubation and reading are done on the same slide.

This communication describes a development of the test described by Boyse *et al.* (1962) in which titration of antiserum, incubation with cells and complement, and reading can all be carried out on one slide. The lack of bias and the reproducibility of this method of titration were tested and the reliability of rapid grading of the tests was assessed.

The paper consists of two parts. The first part is a general description of the materials and methods of the modified cytolytic test, and the second part is the analysis of variance of this method of testing in one clearly defined cytolytic system.

EXPERIMENTAL METHODS AND MATERIALS

Preparation of slides

Eleven rings were ground on the surface of a 7·6 cm. × 5·0 cm. glass microscope slide, the size of each ring being 12·5 mm. internal diameter and 18·7 mm. external diameter. The function of these ground circles was to provide a base to which araldite resin would adhere. The glass was then thoroughly defatted with detergent, rinsed and dried in a 60° C. incubator. Araldite resin was prepared by mixing 100 parts by weight of resin X 83/93 with eight parts by weight of hardener HY 951. The mixture of resin and hardener was applied to the ground rings on the slides from a pasteur pipette; this was best done at 37° C. to obtain a dry slide. To facilitate the application of resin, the slide was clamped to a revolving table and gently rotated whilst the pipette was held in a clamp. After 24 h. at 37° C. the resin had hardened sufficiently for the slides to be washed again and then siliconed after which they were ready for use. Slides prepared in this way can be washed and reused many times.

Since this experiment was performed slides have been obtained from Clay-Adams Inc. New York, U.S.A., which are very similar to the ones described above

having twelve ceramic rings on a 3 in. × 2 in. slide. These when siliconed are quite adequate for this test.

Loops and droppers

Standard droppers to deliver and wire loops to hold 0·025 ml. of saline were obtained from Cooke Engineering Co. Alexandria, Virginia, U.S.A. These were normally supplied for use with a plate micro-titration system developed by Sever (1962).

General description of test

One standard drop (0·025 ml.) of diluent, usually tissue culture medium 199 (Morgan, Morton & Parker, 1950) was placed in each well of the slide. A wire loop (holding 0·025 ml.) was filled with serum and dipped successively into each drop of diluent. Standard mixing was obtained by rotating the loop five times in each drop. The loop and slide are shown in Pl. 1. At the end of a titration the loop was rinsed in a stream of diluent, dried on clean filter paper, and was then ready for the next titration.

The washed cell suspension to be tested was diluted to a concentration of between 5×10^4 and 5×10^5 cells per ml. With large cells the lower concentration was preferable, whereas with small cells such as lymphocytes a concentration slightly in excess of 10^5 cells per ml. proved most suitable. The dilution of complement used varied with the test system, but when using rabbit complement it was usually in the range 1/10 to 1/20. Equal volumes of diluted cell suspension and diluted complement were mixed and one standard drop added to each well. The slide was then incubated in a moist chamber at 37° C. for 90 min.

At the end of incubation the slide was removed from the chamber and a 12·5 mm. diameter round cover-slip was placed on each drop. The slide was then examined under phase-contrast illumination at a magnification of 100 or 250 times. The titration end-point of 50 % cell lysis (CL 50) was estimated from gradings made by assigning each dilution into one of seven grades. Viability was determined by phase-contrast microscopy alone without the use of dyes. This simplified the technique whilst giving the same accuracy as dye exclusion methods. The observation of Goldberg & Green (1959) that cells treated with antibody and complement swelled and their contents became visible under phase microscopy, at the same time as they were losing most of their soluble cytoplasmic contents (Green, Fleischer, Barrow & Goldberg, 1959), provided very adequate differentiation of live and dead cells. Examples of a negative control of cells in the presence of complement but no antibody and one of maximum lysis where cells have been treated with antibody diluted 1/128 and complement are shown in Pl. 2*a* and *b*.

REPRODUCIBILITY EXPERIMENT

General considerations

Errors in the micro-titration system could accumulate in a variety of ways, e.g. from variation in the size of the drops dispensed or from inadequate mixing. If such errors are systematic the results of the titration may be biased. In particular

if mixing is incomplete it will tend to raise the apparent number of dilutions required to reach a given end-point. Errors of all forms might be expected to accumulate as the number of titrations carried out on the slides increased.

In principle, the bias of the micro-titration system may be investigated by testing the same cell suspension with, for example, a 1/2 and 1/32 dilution of the same antiserum made up using standard diluting techniques. If the micro-titration method is unbiased the 1/2 dilution of antiserum should require exactly four more dilution steps to reach the CL50 than the 1/32 antiserum dilution. The magnitude of other errors involved may be estimated by experimental replication.

Detailed considerations

Using these principles an experiment using a standard statistical design was planned as follows:

Five master dilutions (1/2 to 1/32) of an antiserum were prepared from a single source on each of 5 days. Each of these master dilutions was titrated by the micro-titration method described above on five slides. Each day's testing was replicated using a second set of five slides.

The experimental design allowed for the possibility of four factors each at five levels affecting the results, namely: (*a*) the five dilutions of antiserum; (*b*) the individual slides 1–5 or 6–10; (*c*) days; (*d*) order of reading.

These four factors were arranged in a balanced design, namely a Graeco–Latin square (Fisher & Yates, 1953), so that the effect of each factor can be studied independently of the others, The square used for slides 1–5 was exactly replicated using slides 6–10.

Cells and reagents

Cells. A continuous line of mouse thyroid cells C3H-T1 (Franks, Daniel, Gurner & Coombs, 1964) were washed free of growth medium with medium 199 and suspended in a preserving medium of methyl cellulose and dimethyl sulphoxide (Spooner, 1965). Seven drops of cell suspension and preserving medium were distributed to each of ten vials from a single pipette giving a final volume in each of approximately 0·2 ml. These were numbered, and stored in liquid nitrogen. The initial cooling to $-100°$ C. was carried out slowly in an expanded polystyrene plug of the Linde flask (Nagington & Greaves, 1962). In this way standard uniform cell suspensions were available for each set of slides tested each day.

Antiserum. Rabbit antiserum prepared against mouse red cells (with a cytolytic titre of 512 against C3H-T1 cells) was pipetted accurately in 0·5 ml. volumes into each of five siliconed 2·0 ml. tubes. Each was numbered and stored at $-23°$ C. One day's testing with all ten slides was carried out initially to check the system, and one of the five antiserum samples was used up. A further 0·5 ml. of antiserum had to be measured out to replace it. This was done without thawing, pooling and remeasuring the original four. An error led to serum from a different bleeding of the particular rabbit used for antiserum production being used, and the titre of this was higher than in the remaining four antiserum samples. Since a balanced experimental design was used, however, this did not invalidate the results.

Complement. Rabbit serum as a source of complement was pooled and placed in five 0·5 ml. amounts, numbered and stored in liquid nitrogen. A sample of cells and a sample of serum and complement were each removed from storage in random order on each day of testing.

The antiserum was thawed and doubling dilutions of this serum were made in 0·5 ml. amounts using a clean graduated 1 ml. pipette at each dilution step. Dilution was carried out in siliconed tubes so that any fluid on the side of the tube could be included in the titration by brief centrifugation before mixing.

When the master dilutions of antiserum had been made up they were relabelled with numbers 1–5 in accordance with the experimental design and the original labels were removed. The relabelling was done by a member of the laboratory staff not otherwise involved in the experiment, and the procedure was such that those subsequently titrating the antiserum and reading the tests had no idea what the results were likely to be.

The vial of cell suspension was thawed rapidly in a 37° C. water bath and diluted to 2 ml. with medium 199. It was then centrifuged for exactly 10 min. at 60*g* in a 5 cm. × 1 cm. tube, the supernatant was removed and the deposited cells resuspended in exactly 1·5 ml. of medium. The concentration of the final cell suspension from one vial was counted with a Coulter Electronic Cell Counter Model B, using a 100 μ aperture tube, and was found to be $6{\cdot}7 \times 10^4$ cells per ml.

Complement was thawed and diluted 1/12 with medium 199. Complement and antiserum dilutions were kept at 4° C. until required.

Details of the test

The master dilutions of antiserum which had been relabelled with the numbers 1–5 at the beginning of each day's testing were titrated in numerical order on slides 1–5 respectively. Thus because the order of these titrations was the same as that of the slide numbers each day, any effects due to carry-over from one slide to the next were confounded with effects due to different slides. For simplicity of experimental arrangements this shortcoming of the design was accepted. It was expected that neither slides nor effects of carry-over from slide to slide would appreciably affect the results. Then one drop of cell suspension mixed with the complement dilution was applied to each well of the five slides followed by incubation as already described. Half an hour after the first five slides began incubation the test was repeated on slides 6–10. A fresh sample of cell suspension from the original thawed sample was mixed with complement for these repeat titrations. Thus on each day the test procedure was repeated exactly except for a slightly longer time between thawing and testing and a shorter time between the end of incubation and reading in the second group of slides.

After incubation the slides were graded into seven grades by two observers. The grades were as follows: 6, > 99·5 % dead; 5, 99·5 to 90 % dead; 4, 90 to 60 % dead; 3, 60 to 40 % dead; 2, 40 to 10 % dead; 1, 10 to 0·5 % dead; 0, < 0·5 % dead. A third person counted the percentage of dead cells in 100 cells.

The order in which the slides were read within a group of five was determined by the experimental design, and the results of each slide were recorded on separate

slips of paper, stapled together in the order of reading for the particular day. This was a simple way of ensuring that the prescribed order of reading was adhered to and that the observer was minimally affected by earlier readings.

The time and order of grading and counting varied irregularly during the experiment. On occasions the slides stood for up to 2 hr. at room temperature after incubation before they were graded.

Calculation of the end-point of 50 % *cell lysis* (*CL*50)

Cytolytic tests are a form of bioassay, and the number of wells through which an antiserum is titrated before the CL 50 is reached, X, is directly proportional to the $\log_2$ concentration of antiserum in the well. When the percentage of cells killed is

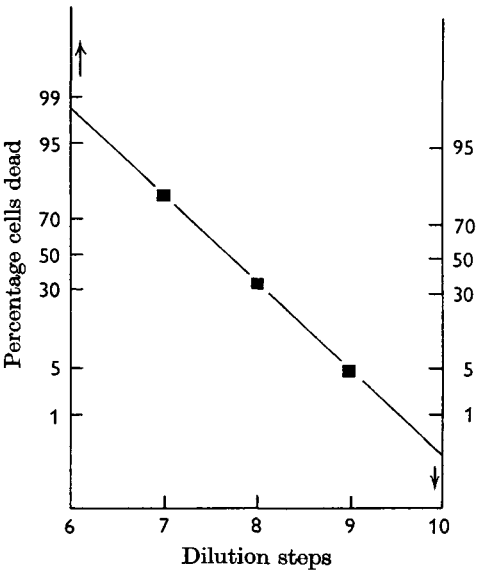


Fig. 1. Results from the first slide encountered with three counts between 95 and 5 % plotted against the number of micro-titration dilution steps on arithmetic probability paper.

plotted on arithmetical probability paper against the $\log_2$ concentration, or equivalently X, the relationship may be expected to be approximately linear from bioassay studies in other fields (Finney, 1947). This is borne out by Fig. 1, which shows the results of the first slide encountered with counts of between 1 and 99 % in three successive wells. The majority of slides gave countable results in only two wells, the remainder of the wells containing 99 % or more live or dead cells.

The CL 50's were estimated by plotting the two counts, on either side of 50 % as in Fig. 2 and reading off the results as the point where the line joining these two points intersects the 50 % line. Similarly, the grades 6 to 0 were taken as corresponding to counts of 99·99, 95, 75, 50, 25, 5 and 0·01 % dead cells respectively. Plotting these percentages on arithmetic probability paper (Fig. 2) and joining all possible pairs of successive grades either side of the 50 % count we find that the grading system groups the result of a titration as a whole number or one of five intermediate values.

https://doi.org/10.1017/S0022172400045253 Published online by Cambridge University Press

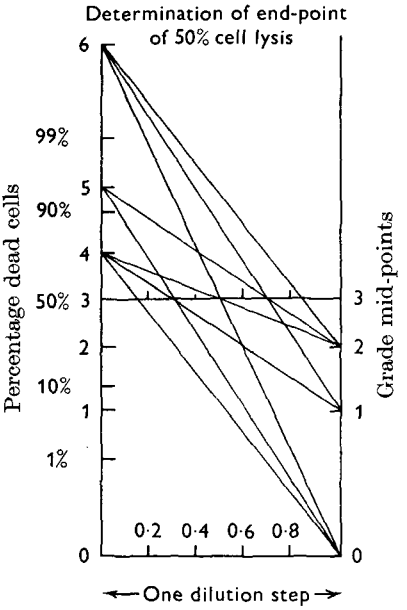


Fig. 2. How the CL 50 is derived. The diagram shows the joining of the mid-points of each grade. To derive the end-point from counted results the exact counts on each side of 50 % are joined.

RESULTS

The CL50's determined from cell counts were used to analyse the reliability, bias and other factors affecting the micro-titration system.

Reliability

The standard deviation (S.D.) of a CL50 estimated by a single micro-titration may be determined from standard analysis of the data. Two estimates of this S.D. were calculated, one from each replication of the experiment. They were 0·14 and 0·3 wells respectively, and are statistically significantly different ($0{\cdot}05 > P > 0{\cdot}025$). Subsequent detailed examination of the data suggested that there might have been two recording errors and that these had inflated the second estimate of the S.D. None of the observations, however, differed very greatly from expectation, and it was felt that if there were any recording errors they were of the kind that might easily occur occasionally in routine experimental work. We therefore pooled all our data and obtained the overall estimate of the S.D. of a single micro-titration as 0·23 dilution step.

In titrating an antiserum experimental errors carry over from one dilution to the next. It seemed important, therefore, to investigate whether the reliability of micro-titration deteriorated with the number of dilutions performed.

Table 1 shows the S.D.'s for a single titration with each master dilution of antiserum, and the nearest whole number of micro-dilutions used to reach the CL50. These S.D.'s were calculated by differencing the two results for the same master dilution obtained each day, and taking out average differences and the overall

https://doi.org/10.1017/S0022172400045253 Published online by Cambridge University Press

effect of slides. As a result the S.D.'s shown in Table 1 are comparable with the overall S.D. shown, but each have only 3 degrees of freedom. They do not differ significantly from one another, nor from the overall S.D. There is no evidence in Table 1 that experimental errors increase significantly with the number of micro-dilutions, and it is concluded that the random experimental errors introduced by this method of making doubling dilutions are small relative to the other errors involved, for example making up the master dilutions, dispensing the drops and counting the cells, etc.

Table 1. *Effect of the number of micro-dilutions on experimental errors*

Concentration of antiserum tested...	1/32	1/16	1/8	1/4	1/2	All concentrations
No. of dilutions to 50 %, end-point = X	4	5	6	7	8	—
S.D. (of X)	0·26	0·14	0·39	0·16	0·29	0·23

Lack of bias

Although the micro-titration method of doubling dilutions may not introduce large experimental errors, unless there is satisfactory mixing in the wells at each step the method will be biased.

Table 2 shows the mean CL50 for each of the five master dilutions of antiserum together with their standard errors. As already explained, if the micro-titration method was unbiased the number of wells to reach the end-point should decrease

Table 2. *The mean no. of micro-dilutions to reach a* 50 % *end-point for each master dilution, and their standard errors*

Master dilution	No. of micro-dilutions to 50 % end-point	
	Mean	S.E.
1/2	8·12	0·023
1/4	7·07	0·023
1/8	6·02	0·023
1/16	5·12	0·023
1/32	3·99	0·023

by exactly 1 each time the master dilution was doubled. The results shown in Table 2 conform closely to this pattern. There is no suggestion that the means are non-linear (see Table 3(*c*)) and the estimated reduction in the number of micro-titrations required for each doubling dilution of the master antiserum is $-1{\cdot}022$ with 95 % confidence interval ($-0{\cdot}975$ to $-1{\cdot}069$) (Fig. 3). Thus the observed relationship is not significantly different from -1, and there is no evidence that the micro-titration method is significantly biased in relation to the method of preparing doubling dilutions of master antiserum.

Differences between slides and between days

Table 3 sets out the analysis of variance of the experimental results. In addition to the significant linear differences between dilutions the table shows that there

https://doi.org/10.1017/S0022172400045253 Published online by Cambridge University Press

Table 3. *The analysis of variance of the experimental observations*

Measured variable: the number of micro-dilutions required to reach a 50% end-point as determined from counted results.

Source of variation	Sum of squares	Degrees of freedom	Mean square	Significance of variance ratio
Replications	1·02	1	(*a*) 1·02	*a*/*g* insignificant
Master dilutions:				
Line fitted to means	104·35	1	(*b*) 104·35	*b*/*r* 0·001 > *P*
Variation of means about fitted line	0·10	3	(*c*) 0·03	*c*/*r* insignificant
Days	15·61	4	(*d*) 3·90	*d*/*r* 0·001 > *P*
Order of reading	0·41	4	(*e*) 0·10	*c*/*r* insignificant
Slides:				
Linear trend, ? due to carry-over effects from one titration to next	0·43	1	(*f*) 0·43	*f*/*g* insignificant
Variations about linear trend	1·50	7	(*g*) 0·21	*g*/*r* 0·001 > *P* > 0·001
Interactions of master dilutions, days and order of reading with replication	0·7	12	(*h*) 0·06	*h*/*r* insignificant
Residual	0·87	16	(*r*) 0·0542	—
Total	124·99	49		

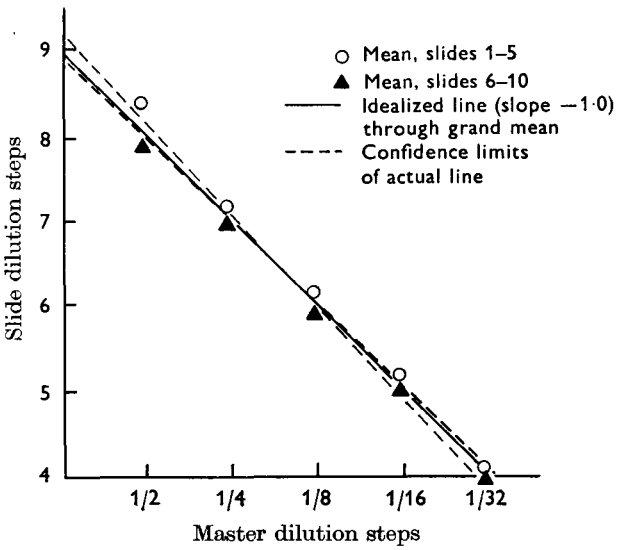


Fig. 3. The means of the CL 50's for each master dilution, i.e. the average end-point over the 5 days for each master dilution when titrated on slides 1–5 and 6–10 plotted against the master dilutions.

were significant differences between slides and differences between days. The order of counting the slides did not affect the results, and the differences between days, between master dilutions and order of reading effects were consistent in both replications of the experiment.

Slides. The differences between slides is illustrated in Fig. 4, in which the average CL 50 over the five master dilutions is shown for each slide. It will be observed

https://doi.org/10.1017/S0022172400045253 Published online by Cambridge University Press

that the average for slides 1–5 is higher than for slides 6–10. This difference corresponds to the sum of squares for replications which, compared with differences between the slides, is not significant. A probable explanation, discussed in the next section, is that the second set of slides stood for a shorter time on the bench after incubation.

It will be noted from Fig. 4 that the CL50's for slide 10 were on average 0·25 dilution higher than those of slides 1–5 and 0·5 dilution higher than those of slides 6–9. Before the experiment began it was noted that slide 10 was not as well siliconed as the others. It was included to see what effect this might have on the results. The higher result for slide 10 shown in Fig. 5 could be explained by the fact that poor siliconing caused incomplete mixing and consequently a higher end-point.

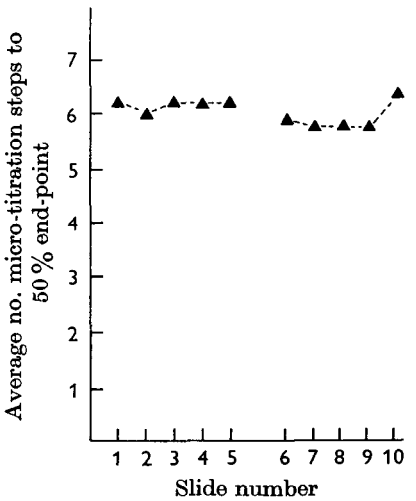


Fig. 4. The mean of the results for each slide, to show the overall effect of each slide.

Relative to the variation from slide to slide, there is no evidence of a trend within a set of 5 suggesting a carry-over effect from one micro-titration to the next.

Days. The highly significant difference between days shown in Table 3 largely results from the fact that the average results on the last day were more than a dilution higher than on any of the previous four days. The averages for the first 4 days also vary significantly, $0{\cdot}05 > P > 0{\cdot}01$ compared with residual variation, but the range of their variation is 0·33 dilution and, compared with the variation in the day-to-day means, the results for the 5th day are statistically significantly different from the average of the other four, $0{\cdot}05 > P > 0{\cdot}01$.

The fact that there are day effects which are consistent in both replications suggests that the master antiserum may have been made up slightly differently on each of the first 4 days rather than that there were differences between the stored cell samples or complement, of which different samples were used for slides 1–5 and 6–10 each day. Another source of day-to-day variation was the length of time the slides stood after incubation before they were counted. This period though shorter for slides 6–10 because they were read immediately after 1–5

varied similarly from day to day for both sets of slides. Variation in standing time cannot explain the generally high results on the last day.

Estimating the CL50 by grading

At an early stage of the experiment it was noticed that the end-point changed after the slide had been standing on the laboratory bench for some while. The time interval between grading and counting the results was then deliberately varied to study this.

The time taken to grade or count a set of five slides is negligible. All slides were graded by observers I and II and counted by observer III. Fig. 5*a* shows, for each set of five slides, the average end-point difference between observer I's graded

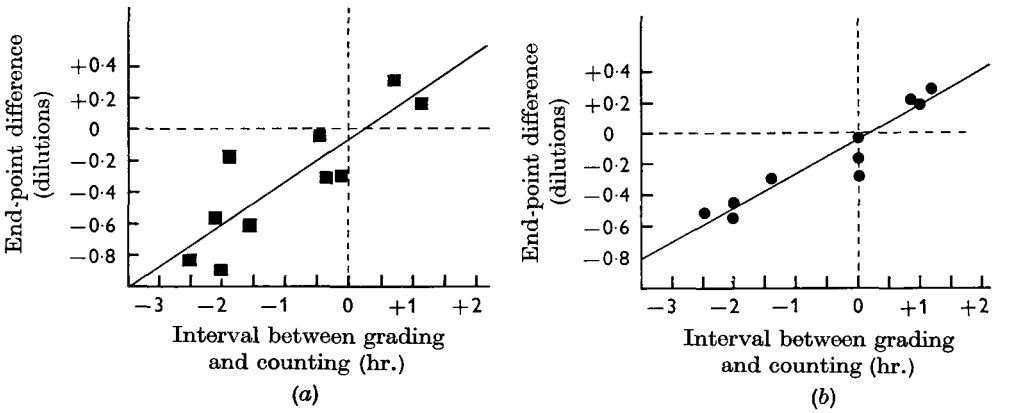


Fig. 5. Difference between graded results and counted results plotted again time interval between grading and counting. (*a*) Observer I. (*b*) Observer II.

results and observer III's counted results plotted against the time the slides stood between grading and counting. Similarly, Fig. 5*b* is a plot of the average end-point difference between observer II's grading and observer III's counting, against the time interval between grading and counting.

Both sets of results show that the end-point advanced with time, the rates of advance were similar in both cases and were of the order of a dilution every 4 or 5 hr.

The regression lines shown in Fig. 5*a* and *b* indicate the estimated relationship between the end-point difference between grading and counting on the one hand and the time interval between grading and counting on the other. The bias of grading as compared with counting is the end-point difference when the time interval between grading and counting is zero. The fitted regression lines are not significantly different from zero at this point, from which we conclude that there is no evidence that assessing the end-point by grading gives rise to biased results.

The standard deviation of a CL50 determined by counting was estimated to be 0·23 dilution. Using this estimate, we obtain from regression analysis to fit the lines shown in Figs. 5*a* and 5*b*, that the S.D. of a CL50 estimated by grading is 0·25 for observer I and 0·17 for observer II. The reliability of grading is, therefore, very similar to that of counting.

https://doi.org/10.1017/S0022172400045253 Published online by Cambridge University Press

DISCUSSION

The use of standard micro-titration equipment for the performance of cytolytic tests on a siliconed glass slide provides considerable simplification of technique from previous methods.

In the test described here incubation was carried out on the slide without a cover-slip and the cells settled within the restricted area of the base of the drop. When, before reading, a cover-slip was applied very few of the cells were disturbed, whether live or dead, and hence most of the cells in the test could be seen under one field of the $\times 10$ objective. This meant that tests could be carried out with as few as 200 to 300 cells per drop, although usually about 500 cells per drop were employed. Before the test could be accepted it was felt that the reliability and bias of the method should be investigated.

Reliability. The standard deviation of a single estimate of a CL50 which was found to be 0·23 dilution, was derived from cell counts of more than 100 cells in at least two wells. Although only a sample of cells was counted, calculations indicate that random sampling errors could account for less than half the observed S.D.

In grading the results there will be classification errors. It is possible, however, that the method admits of a scan of a larger number of cells, and hence a more accurate impression of the mortality than might be obtained by counting cells in a limited field. In addition, the graded results allow only six intermediate end-points between whole dilutions, but random errors introduced by this grouping can be shown to be relatively small.

The estimates given for the S.D. for a single titration all assume that repeated estimates are made on the same day with the same slide and using the same cells and antiserum. It will be noted that the slides vary slightly one from another, that the results appear to vary from day to day, and that the time the slides are left standing affects the end-point.

It may be concluded, therefore, that in making any comparison involving differences of less than one dilution, experimental replication is essential, preferably on different days, and also that it is essential to standardize the amount of time slides are left standing before they are counted and to see that the slides themselves are constantly maintained fully siliconed.

Bias. From the results of this experiment no evidence was found that the micro-titration method is significantly biased, compared with doubling dilutions made in 0·5 ml. amounts with graduated pipettes. The observed results predict a bias of 0·2 dilution in 10 but do not exclude the possibility that the bias could vary between $-0{\cdot}25$ and $+0{\cdot}79$ dilution in 10. Thus whilst the limits of bias at each step are fairly small the possibility of some bias building up by the last well on the slide has not been excluded. The experiment could have been more sensitive in this respect if a wider range of master dilutions had been used.

Suitability of grading. It can be seen that CL50 estimated by grading is not biased relative to the counted results, and also that the S.D. of graded results is very similar to that of counted results; from this it is concluded that grading is an efficient

method of assessing a CL50. Because grading is very much quicker than counting it has been adopted for all subsequent cytolytic tests.

The micro-titration method described is simple, reliable and unbiased. It has made it possible to do much more extensive cytolytic tests with known accuracy than was possible with previous methods of testing. The ability to use stored cells in cytolytic tests (Spooner, 1965), together with this method of titration, means that a standard cell suspension can be available for the comparison of antisera on different days. Standardization of incubation and reading coupled with repetition of the test if small differences are to be detected, is necessary.

SUMMARY

A method of micro-titration for cytolytic antibodies is described. Standard drops of diluent are placed on a subdivided siliconed slide and dilutions are made by rotating a wire loop holding 1 drop of serum in each drop sequentially. One drop of a mixture of cells and complement is then added. The slide is incubated in a moist chamber for 90 min at 37° C. after which a small cover-slip is placed on each drop and the amount of cell lysis, judged by phase-contrast microscopy, is graded.

An analysis of variance of the method has been performed and the method has been found to be reliable, accurate and unbiased.

It allows accurate estimates of cytolytic activity in sera using cell concentrations of 10^4 to 10^5 cells per ml. Eight ten-dilution titrations can be carried out with 1 ml. of such a suspension.

We would like to thank Miss Lynn L. Flory and Dr David Franks for most valuable assistance in the grading and counting of the slides. Also we are grateful to Dr M. R. Daniel for supplying the C3H-T1 cells.

One of us (R.L.S.) was a Research Training Scholar of the Animal Health Trust whilst this work was done.

We would also like to thank Dr R. R. A. Coombs for his constant help and encouragement.

REFERENCES

Boyse, E. A., Old, L. J. & Thomas, G. (1962). A report on some observations with a simplified cytotoxic test. *Transplantn Bull.* **9**, 435.

Finney, D. J. (1947). *Probit Analysis.* Cambridge University Press.

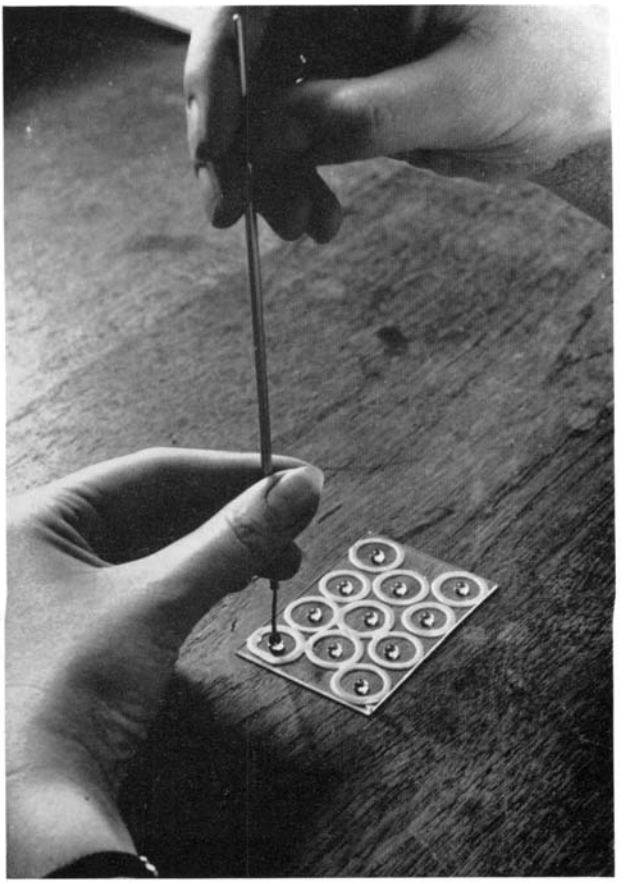
Fisher, R. A. & Yates, F. (1953). *Statistical Tables for Biological, Agricultural and Medical Research.* London: Oliver and Boyd.

Franks, D., Daniel, M. R., Gurner, B. W. & Coombs, R. R. A. (1964). Variation of the Forssman antigen on cells in culture. *Expl Cell Res.* **36**, 310.

Goldberg, B. & Green, H. (1959). The cytotoxic action of immune gamma globulin and complement on Krebs ascites tumour cells. I. Ultrastructural studies. *J. exp. Med.* **109**, 505.

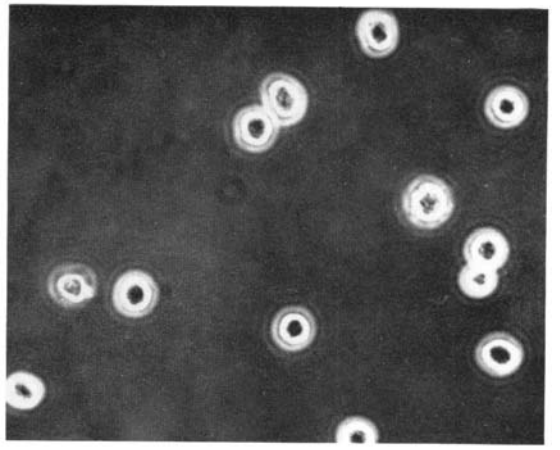
Gorer, P. A. & O'Gorman, P. (1956). The cytotoxic activity of isoantibodies in mice. *Transplantn Bull.* **3**, 142.

Green, H., Fleischer, R. A., Barrow, P. & Goldberg, B. (1959). The cytotoxic action of immune gamma globulin and complement on Krebs ascites tumour cells. II. Chemical studies. *J. exp. Med.* **109**, 511.

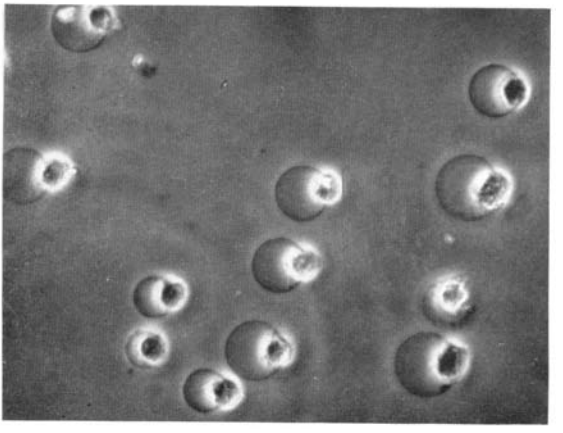


R. L. SPOONER, F. W. BOWDEN AND R. G. CARPENTER (*Facing p.* 380)

https://doi.org/10.1017/S0022172400045253 Published online by Cambridge University Press



(*a*)



(*b*)

https://doi.org/10.1017/S0022172400045253 Published online by Cambridge University Press

HANKS, J. M. & WALLACE, J. H. (1958). Determination of cell viability. *Proc. Soc. exp. Biol. Med.* **98**, 188.

MORGAN, J. F., MORTON, H. J. & PARKER, R. C. (1950). Nutrition of animal cells in tissue culture. I. Initial studies on a synthetic medium. *Proc. Soc. exp. Biol. Med.* **73**, 1.

NAGINGTON, J. & GREAVES, R. I. N. (1962). The preservation of tissue culture cells in liquid nitrogen. *Nature, Lond.*, **194**, 993.

REIF, A. E. & NORRIS, J. H. (1960). A system for quantitative determination of cytotoxic activity of antisera to ascites tumour cells. *Cancer Res.* **20**, 1235.

SEVER, J. L. (1962). Application of a microtechnique to viral serological investigations. *J. Immunol.* **88**, 320.

SPOONER, R. L. (1965). The use of preserved cells in immune cytolytic tests. *Expl Cell Res.* **38**, 465.

EXPLANATION OF PLATES

PLATE 1

Subdivided slide with drops of diluent in place and loop as held during titration.

PLATE 2

(*a*) Preserved C3H–T1 cells; control suspension after 90 min. incubation with 1/24 rabbit complement.

(*b*) Preserved C3H–T1 cells; complete lysis in presence of 1/24 rabbit complement and 1/128 dilution of a rabbit antiserum prepared against BALB/c red cells.