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A comparative study of a plaque and quantal method for assaying the neutralizing activity of antisera to type 1 poliovirus*

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INTRODUCTION

The plaque neutralization test was originally developed for assaying antisera to bacteriophage, and was later adapted to the measurement of the neutralizing antibody content of sera against various animal viruses, such as poliovirus (Dulbecco, Vogt & Strickland, 1956). Before this, except for those viruses which produce pocks on the chorio-allantoic membrane of the developing chick embryo, neutralizing activity had been estimated by the classical 'quantal' method, using either eggs, animals, or tubes of tissue culture.

While it is intuitively recognizable that the plaque neutralization method is probably more sensitive than the quantal in all circumstances, the quantitative relationship between the results obtained by these two methods, for any one system, has never been fully elucidated. Moreover, it has always been tacitly assumed that either of these two assay techniques gives a linear response with respect to the concentration of neutralizing antibody. This latter assumption could only be tested rigorously in the case of either method, provided that some entirely independent means of measuring antibody concentration was available. At the same time, it can be shown that if both the quantal and plaque methods independently yield linear estimates of the neutralizing antibody concentration, then the two assays themselves will be linearly related.

During the study of the antibody response of the rabbit to type 1 poliovirus, the neutralizing antibody content of a number of sera from sample bleedings was estimated in parallel by the quantal and plaque neutralization techniques. The two techniques were compared for accuracy, sensitivity and facility of use under ordinary laboratory conditions. In addition, a comparison between the antibody estimates yielded by the two methods was carried out in order to confirm that the relationship between them is of the general linear form

$$p = Rq + c,$$

where p is the plaque neutralizing titre, and q the quantal neutralizing titre, for each serum in the series, R and c being constants.

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MATERIALS AND METHODS

Materials

Virus

The virus challenge used in both neutralization tests was the Brunhilde strain of type 1 poliovirus grown in the ERK line of HeLa cells (Westwood, Macpherson & Titmuss, 1957), by the technique of Churcher, Sheffield & Smith (1959). The tissue culture fluid produced was stored at $-10°$ C., except for the sample in current use which was stored at 4° C.

Sera

Two rabbits, 1/59 and 2/59, were immunized with the Brunhilde strain of type 1 poliovirus grown in ERK, concentrated and partially purified by high-speed centrifugation, so that it contained approximately $10^{9\cdot5}$ plaque-forming units (pfu) per ml. Each rabbit received 0·5 ml. of this antigen intravenously on day 0, 5 ml. on day 20 and 2·5 ml. on day 127. A total of thirty blood samples from each rabbit was taken at intervals over a period of 217 days after the initial inoculation. The serum was separated, inactivated by heating at 56° C. for 30 min., and stored at $-10°$ C. A pool of sera from the primary and secondary responses of rabbits similarly immunized was used as a reference standard serum.

Cell cultures and media

The ERK line was used to estimate both virus and residual virus concentration. The cells were cultured in a growth medium consisting of Earle's balanced salt solution with 0·25 % lactalbumin hydrolysate, 5 % tryptic digest meat broth, and 10 % calf serum. To each 100 ml. of medium, 2 ml. of 2·78 % sodium carbonate was added and the pH adjusted to 7·4 by gassing the medium with CO_2 immediately before use. Penicillin and streptomycin were added to the final medium to give a concentration of 100 units/ml. and 100 μg./ml. respectively.

For plaque production, the cells from a confluent cell sheet on one side of a 250 ml. 6-sided Pyrex bottle (approximately 4×10^6 cells) stripped by versene, were seeded into three bottles in 10 ml. of growth medium per bottle, and usually produced confluent monolayers in 48 hr.

For tube cultures, the cells from a similar confluent cell sheet (approximately 4×10^6 cells) were seeded into twenty $6 \times \frac{5}{8}$ in. tubes, using 1 ml. of growth medium per tube. Seeding at this concentration gave rise to confluent growth in 48 hr.

Methods

All dilutions were made in physiological saline with 10 % calf serum added. Dilutions and inoculations were made with mercury calibrated pipettes, using volumes of 0·1 ml. or greater.

Virus titration by the plaque method

The technique employed was based on that described by Hsiung & Melnick (1957), 0·3 or 0·5 $\log_{10}$ dilutions of virus suspension were made and 0·1 ml. of each dilution delivered on to each of three cell monolayers previously drained of growth medium. One hour at 37° C. was allowed for adsorption of the virus and then

10 ml. of overlay, held at 43° C., was added to each bottle, mixed with the inoculum and allowed to cover the cell monolayer. This overlay consisted of equal parts of 2·4 % Difco 'Bacto' agar and double strength medium with increased sodium carbonate. The sodium carbonate concentration of the complete overlay was 0·11 %, the double-strength medium which was not gassed contained 0·22 % sodium carbonate. An hour at room temperature permitted the agar to harden, after which time the bottles were inverted and the cultures incubated at 37° C. After 42 hr. 0·25 ml. of 1/1000 neutral red was flooded over the surface of the agar and the bottles immediately inverted and re-incubated at 37° C. Plaques could be discerned 5 hr. after the addition of the dye, when the site of each plaque was marked on the bottle. The bottles were examined on each of the following 2 days when plaques making their first appearance were marked and the total number of plaques present counted, a final count of the plaques being recorded on the 4th day. The mean of the plaque counts of three bottles at any one dilution gave an estimate of the number of plaque-forming units per ml. of the original virus suspension when corrected for the dilution and volume of the inoculum.

Plaque neutralization test

Dilutions of serum at 0·3 $\log_{10}$ intervals were made in 0·5 ml. amounts on either side of the dilution which gave a 50 % reduction in plaque count of the virus challenge, as assessed in a pilot experiment using 0·5 $\log_{10}$ dilutions. Each dilution was mixed with an equal volume of virus suspension containing 40 pfu/0·1 ml.; one tube containing diluent in place of the serum dilution was employed to assay the virus challenge. After 1 hr. incubation at 37° C., the virus-serum mixture for each serum dilution and the virus challenge was inoculated into three bottles, 0·2 ml. per bottle. The plaque count at each serum dilution was determined by the procedure described under virus titration and the neutralizing titre of the serum was taken as the reciprocal of the initial serum dilution which reduced the plaque count to 50 % of the virus challenge. Percentage neutralization at each serum dilution, calculated as a mean of the three bottles, was plotted against $\log_{10}$ of the serum dilution, and the 50 % neutralization end-point calculated from a straight line fitted to the resulting experimental points by the method of least squares.

Virus titration by the quantal method

A volume of 0·1 ml. of each 0·3 $\log_{10}$ or 0·5 $\log_{10}$ dilution of virus suspension was inoculated into each of six tube cultures containing 2 ml. of maintenance medium. Maintenance medium differed from growth medium only in an increase of sodium carbonate from 2 to 3 ml./100 ml. of medium and the omission of gassing. The tubes were rolled at 37° C., read at 24 hr. to detect any toxic effects, and subsequently at 3, 4, and 5 days, the results recorded being those read on the 5th day. The titre, expressed in TCD 50's, was taken as the reciprocal of that dilution of virus suspension which produced a cytopathogenic effect in half the tubes inoculated, and was interpolated where necessary by the moving average method of Thomson (1947).

Quantal neutralization test

Dilutions of serum at 0·3 $\log_{10}$ intervals were made in 0·5 ml. amounts straddling the dilution which would protect 50% of the tube cultures exposed to a virus challenge of approximately 100 TCD50. The relevant dilution interval in which the end-point would be expected had been estimated previously as in the plaque neutralization method. Each serum dilution was mixed with an equal volume of virus suspension containing approximately 200 TCD50 per 0·1 ml. and then incubated for 1 hr. at 37° C. The virus challenge was titrated by $\log_{10}$ steps under the same conditions as the serum dilutions. After neutralization had been allowed to proceed for 1 hr. at 37° C., 0·1 ml. of virus-serum mixture was inoculated into each tissue culture tube, six tubes being used per serum dilution. The presence of infective virus at each serum dilution was determined in the manner described under virus titration by the quantal method. The neutralizing titre of the serum was taken as the reciprocal of the initial dilution of the serum which would protect half the tissue cultures against the cytopathic effect of the virus challenge and was calculated by the moving average method. The standard rabbit type 1 antiserum was titrated in the above manner with each batch of serum assays.

RESULTS

The plaque neutralization test

The neutralizing titre of a serum has been defined as the reciprocal of that dilution which will reduce the mean plaque count to half its expected value as determined by the mean plaque count in the absence of serum. This means that the variation of plaque number in individual monolayers is the factor governing the reproducibility of the test.

It will be apparent that the factors limiting the accurate estimation of plaque-forming units per bottle will be: (1) the number of plaques present, (2) their rate of growth, (3) the time for which they are allowed to grow, and (4) the distribution of the infective foci over the cell monolayer. Thus, should two adjacent plaques be in contact when first observed, difficulty will be experienced in distinguishing them subsequently when they enlarge. The following experiments were designed to demonstrate the influence of such coalescence upon the results obtained.

Study of plaque development

Presence of small and large plaques

Although the predominant plaque type was large in both virus titrations and serum assays, small plaques were also present. The possibility that these small plaques arose from a small plaque variant was investigated, in case they represented a genetically stable variant with a different antibody sensitivity. A large and a small plaque were 'picked' and passed in tissue culture. When the cultures showed complete degeneration, each fluid was titrated by the plaque method. The proportion of large to small plaques arising from the large plaque parent was 3·96 to 1, while from the small plaque parent the ratio was 3·63 to 1. As the two ratios

are of the same order of magnitude, the difference in size was not due to genetically different particles, but probably to asynchrony of infection, maturation or release of the virus from the infected cells.

Coalescence of plaques

The inclusion of neutral red in the overlay in virus titrations by the plaque method was found by Darnell, Lockhart & Sawyer (1958) to reduce the plaque count of type 1 poliovirus by 50 %, when this virus was titrated in HeLa cells. It was decided, therefore, to delay the addition of the vital stain until 48 hr. after the inoculation of the tissue culture. It was then possible to mark the plaques as they appeared and to reduce the number which would be lost, through coalescence of enlarging adjacent foci, if counting was delayed until the 3rd or 4th day. To

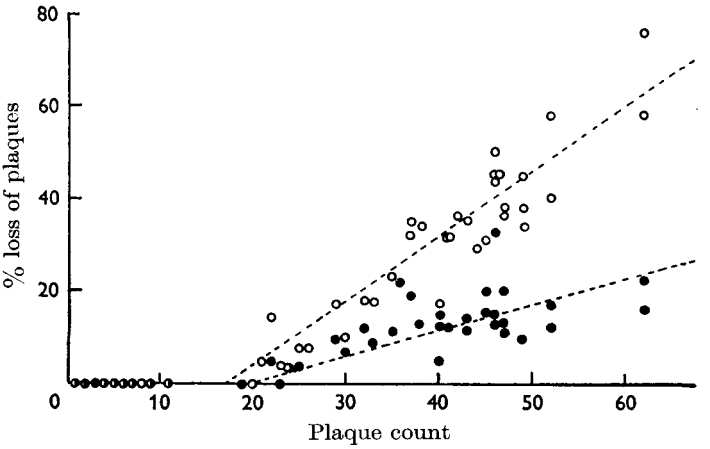

Fig. 1. Percentage loss of plaques due to coalescence as a function of the plaque count. ●, Between 2 and 3 days; ○, between 2 and 4 days.

determine the extent of this loss, several titrations of the standard serum and virus challenge were performed, the plaques being marked on the 2nd, 3rd and 4th days. The number of plaques so estimated (a_2, a_3, and a_4) were then compared with the apparent number of plaques (b_3, b_4) present on the 3rd and 4th days as estimated by ignoring the markings. The percentage loss of plaques at 3 and 4 days (e.g. at 3 days, $[(a_3-b_3)/a_3] \times 100$) was plotted against the plaque count on the corresponding day as shown in Fig. 1. It will be noted that for a plaque count of 20 or less there is little or no loss. Above this figure, for every increase of 10 in the plaque count, there is an approximate increase of 5 % in the loss due to a coalescence between the 2nd and 3rd days, and this is increased to approximately 14 % for the interval between 2 and 4 days. Despite this early marking of infected foci, it is still possible that some plaques were being overlooked because they occurred under pre-existing lesions, or multiple infection of a cell had occurred.

To determine the extent of this loss, several virus titrations were undertaken, and the mean plaque count at each dilution recorded. A loss due to the above-mentioned factors would be expected to follow a similar pattern to that of Fig. 1,

i.e. with a progressive increase in the concentration of virus there would be increased likelihood of overlaying or multiplicity of infection of cells, and a corresponding decrease in the plaque count below that expected. Fig. 2 shows the relationship between virus concentration and the plaque count for four such virus titrations, and there is no evidence that there is any departure from linearity. As the virus challenge seldom exceeded 50 plaques per bottle in the neutralization tests, loss due to this cause can be ignored in this study.

Further evidence that this loss is negligible is found in the effect of the size of the virus challenge upon the titre of an immune serum. If with increasing plaque counts there is loss due to 'overlaying' of plaques, or multiple infection of cells, then with a small number of plaques, i.e. a high degree of neutralization, there will

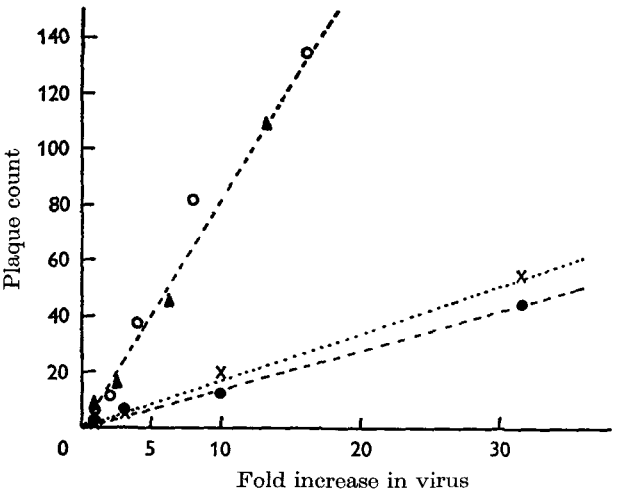

Fig. 2. The relationship between plaque count and virus concentration. ○, ▲, Two titrations of type I poliovirus preparation, 29/59; ×, ●, titrations of two other type I poliovirus preparations, 3/59 and 17/59.

be little or no loss of plaques. However, as the plaque count increases with decreasing neutralization, the plaque loss would correspondingly increase, and be maximal in the virus control. This will obviously not affect each serum dilution equally. Thus not only will the titre be changed, but also the slope of the line relating percentage neutralization to $\log_{10}$ serum dilution. The exact manner in which this affects the neutralization curve can be seen from an experiment where the plaque counts on the 4th day, obtained by daily markings, were compared with those estimated ignoring the previous markings (see Table 1). It can be seen that the titre of the serum is reduced by $0{\cdot}2\log_{10}$, owing to the significant ($0{\cdot}05 > P > 0{\cdot}02$) increase in the slope of the line relating percentage neutralization to $\log_{10}$ serum dilution.

In the example shown, the loss of plaques is overcome by early marking, but should further loss of plaques occur which is not detected by this method, then a reduction in titre with increased virus challenge would be expected. It can be shown, however (see 'Effect of the size of the virus challenge', p. 319) that no such

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relationship is demonstrable. Further evidence in favour of such a loss would be a significant increase in the slope of the line relating percentage neutralization to $\log_{10}$ serum dilution, b, with increase in virus challenge. In a series of ten titrations it was shown that b was not correlated with the size of virus challenge ($0{\cdot}2 > P > 0{\cdot}1$) so that there is no evidence for a residual, significant loss of plaque number when marking at 48 hr.

The reduction in titre and change in slope which are associated with loss of plaques due to coalescence is of considerable importance in those tests where the addition of vital dye is delayed until the 4th day, for then the effect of the size of the virus challenge will be appreciable.

Table 1. *The effect of coalescence of adjacent plaques on the slope of the neutralization curve and the serum titre*

	4th day counts observing daily markings				4th day counts ignoring markings			
Serum dilution	Mean plaque count	Neutralization (%)	'b'	Neutralizing titre	Mean plaque count	Neutralization (%)	'b'	Neutralizing titre
$10^{-3.7}$	1·33	97·5	87	$10^{4.31}$	1·33	95	111·67	$10^{4.1}$
$10^{-4.0}$	8·33	84·5			8·33	68		
$10^{-4.3}$	23·33	56·5			21·33	18		
$10^{-4.6}$	46	14·5			31·33	0		
$10^{-4.9}$	52·67	2			25·67	1		
V.C.	53·67	—			26	—		

'b' = slope of the line relating % neutralization to $\log_{10}$ serum dilution. V.C. = virus challenge.

Error of plaque counting

Many workers have shown that the pock counts on the chorio-allantoic membrane of the developing chick embryo, when titrating pox virus suspensions (Fenner & McIntyre, 1956; Kaplan & Belyavin, 1957; Armitage, 1957; Westwood, Phipps & Boulter, 1957), or virus-serum mixtures (McCarthy, Downie & Armitage, 1958) have a variation greater than that expected if the distribution of the pocks between the membranes was poissonian.

If plaque counts have a Poisson distribution, then the variance between replicate plaque counts would approximate to their means. A mean was calculated for each group of three counts in the plaque neutralization tests on 61 sera. Each mean count was plotted against its own standard deviation, s, in Fig. 3. It will be seen that there appear to be two populations, one tending to a Poisson distribution, inasmuch as the standard deviation approximates to the square root of the mean plaque count, the other having a greater variance. On studying the nature of the sera showing the higher variance, it became apparent that the majority of them were derived from the rabbits which had received only one inoculation of antigen. When the data derived from the sera of the primary responses are plotted independently, the same bimodal distribution is seen, and no further division can be made on the basis of individual rabbits, or on the subculture of the cell line on which the results were obtained. The data from the remaining sera approximate

to a Poisson distribution, as indicated by the relationship between the variance and the mean plaque count. The points obtained from three bottle estimates in virus titrations were also shown to follow the same pattern. From this it can be assumed that the distribution of the plaque-forming elements is the same whether they are taken from virus or serum-virus mixtures. The large degree of scatter of the points around the line predicted by a Poisson distribution could be attributed to the small number of samples from which the statistics have been calculated. As the mean plaque count increases, it would be expected that the distribution would approximate to a normal one, but it is not possible, owing to the scatter of the points, to decide whether the variance is dependent on the mean or not, at higher plaque counts.

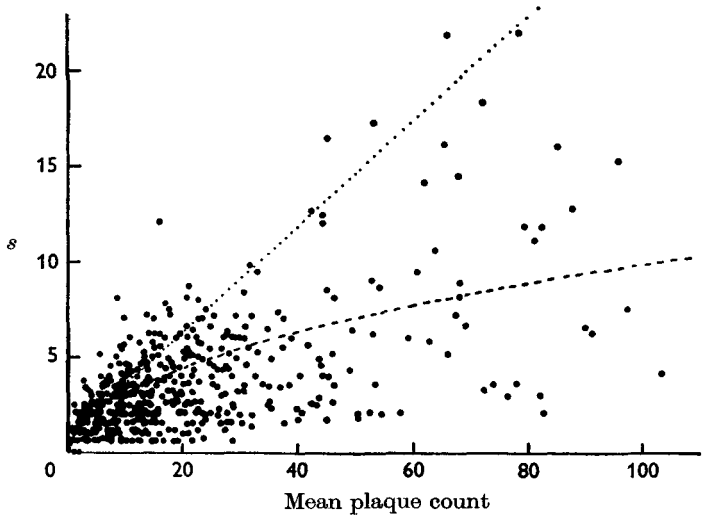

Fig. 3. The relationship between the calculated standard deviation, s, and the mean plaque count for all serum titrations. - - - -, Theoretical relationship for a Poisson distribution; ....., relationship of a group of points with greater values for s than expected from a Poisson distribution.

Error of the plaque neutralization test

In measuring neutralizing antibody titres of the sera from rabbits immunized with poliovirus, it is important to know the error of estimation. As has already been intimated, this will arise directly from the actual variation in plaque count between replicates, and the magnitude of its effect on the calculated neutralizing titres can be directly estimated by repeated titrations.

The standard serum was estimated on one occasion by three replicate titrations, using the same virus challenge, and on seven other occasions with an independent virus challenge (Table 2). This was because the number of replicate titrations that can be accomplished on one occasion is limited by technical factors. A smaller number of estimates were made on the test sera, a primary bleeding 'D' and a tertiary bleeding 'V' and these are also recorded and analysed in this table.

From simple inspection of the data in Table 2, it will be seen that the variation in titre of the standard serum is approximately the same whether the serum is

estimated in three replicate titrations with a common virus challenge (first three rows), or on separate occasions with an independent virus challenge. From this it can be assumed that there was no gross change in the quantity or nature of the antibody, or the behaviour of the tissue culture cells over the period during which these estimations were made, any error being due to the manipulations of the technique itself, e.g. sampling, dilution, counting, etc.

Table 2. *The error of estimating the neutralizing titre of three sera by the plaque method*

Serum	Estimate	Virus challenge (pfu's)	Titre	Mean titre	Standard deviation	Coefficient of variation	Fiducial limits ($P = 0{\cdot}05$)
'Standard'	1	21·33	21,380	22,811	± 3,280	14·38	14,398–30,224
	2	21·33	29,854				
	3	21·33	22,856				
	4	23·67	20,989				
	5	25	20,701				
	6	32·33	28,445				
	7	46·33	22,131				
	8	48·67	21,380				
	9	53·67	20,417				
	10	58·67	19,953				
'D'	1	—	169	206·75	± 26·3	12·72	123–290·5
	2	—	219				
	3	—	227				
	4	—	212				
'V'	1	—	153,780	150,598	± 19,690	13·2	100,298–200,898
	2	—	153,640				
	3	—	162,180				
	4	—	169,820				
	5	—	151,710				
	6	—	112,460				

pfu = plaque-forming unit.

Reference to Table 2 shows that the three sera, having titres between 207 and 150,598 and derived from primary, secondary and tertiary responses, yield a variance between replicates proportional to their mean titre, the coefficient of variation being approximately constant. The overall data show that 95 % of estimates of the titre of any one serum could be expected to fall within 0·4 $\log_{10}$ of each other. These findings are in agreement with French, Armstrong & Nagler (1959), who, using their plaque neutralization technique, determined a coefficient of variation for type 1 poliovirus neutralization of 12·3 %.

Effect of the size of the virus challenge

It would be expected that with an increase in the virus challenge the titre of the serum would be reduced. However, the range of virus challenges that can be studied has strict limitations. A virus challenge of less than 20 pfu results in an unsatisfactory neutralization curve, and in excess of 60 pfu difficulty is experienced in counting the plaques formed. The ten estimates of the standard serum recorded

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in Table 2 have been accomplished with virus challenges in this range. The slope of the straight line of best fit, as calculated by the method of least squares, to the plot of the titre versus the virus challenge does not differ significantly from zero ($P = 0{\cdot}2$). These observations, therefore, show that the virus challenge and the serum titre appear to be unrelated over the range employed. In other words, the change in serum titre with virus challenge is no greater than the variation inherent in the neutralization test itself over the narrow range of virus challenge capable of being studied.

The quantal neutralization test

As each tube in this type of neutralization test does not give an individual estimate, the error of this test and the influence of the size of the virus challenge could be studied directly.

Error of the quantal neutralization test

Twenty-two estimates of the neutralizing activity of the standard serum were made on separate occasions by this technique and the results are recorded in Table 3. Unlike the plaque test, many replicate titrations could be performed by

Table 3. *The error of estimating the neutralizing titre of two sera by the quantal method*

Serum	Estimate	Titre	Mean titre	Standard deviation	Coefficient of variation	Fiducial limits ($P = 0{\cdot}05$)
'Standard'	1	5,370	5,197	± 875·2	16·84	3,377–7,017
	2	6,310				
	3	5,012				
	4	4,571				
	5	5,623				
	6	4,571				
	7	5,012				
	8	6,310				
	9	5,012				
	10	4,571				
	11	6,310				
	12	6.310				
	13	5,012				
	14	4,571				
	15	3,981				
	16	3,350				
	17	5,012				
	18	4,467				
	19	5,012				
	20	5,623				
	21	7,079				
	22	5,248				
'V'	1	28,150	31,058	± 5,254	16·92	14,351–47,766
	2	25,119				
	3	35,481				
	4	35,481				

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the quantal method at any one time. However, to obtain an estimate of the error of this test, which was comparable with that already determined for the plaque test, repeated titrations on different occasions were undertaken. The standard deviation, coefficient of variation and the fiducial limits of the titre of the standard serum calculated from these titrations are also included in Table 3, as is a similar analysis on a serum from a tertiary response, bleeding 'V'. The coefficients of variation are of the same order of magnitude for the standard serum and the high titred serum from the tertiary response, showing that, as in the plaque neutralization test, the error is proportional to the titre.

Effect of the size of the virus challenge on the serum titre

It was possible in the quantal technique to determine the effect of the size of the virus challenge on the neutralizing titre of a serum over a larger range of values than in the plaque neutralization test. The limiting factors operative in the plaque test were no longer applicable, provided that the virus challenge was of such magnitude that the possibility of a tube containing no antibody being uninfected, owing to sampling error, was exceedingly small. The plot of the percentage neutralization against $\log_{10}$ serum dilution, for three titrations of the standard serum against fourfold increase in virus challenge (42, 169 and 676 TCD 50) showed that the slope differed only slightly, but a reduction in the titre with increase in the virus challenge was observed. The relationship between the size of the virus challenge and the corresponding neutralizing titre was such that for every increase of 100 TCD 50 in the virus challenge, within the range studied, the titre of the serum fell by approximately 0·1 $\log_{10}$.

Comparison of the titres of immune sera measured by the plaque and quantal neutralization tests

Each of the neutralization methods used in these experiments yields an estimate of the neutralizing antibody content of a serum in terms of certain numerical parameters p and q, p being the neutralization titre as measured by the plaque method, and q the corresponding neutralizing titre as estimated by the quantal titration. If both p and q are linear functions of the same antibody concentration, then these parameters should themselves be linearly correlated. However, reference to Fig. 4 shows that there is a non-linear relationship between the titres obtained by the two tests, for the bleedings from the secondary and tertiary responses of rabbit 1/59. A good fit to the points obtained has been accomplished by using an arbitrary quadratic equation having the form $p = 8{\cdot}2q - 0{\cdot}12q^2$. A similar good fit has been obtained in the case of the primary bleedings from both rabbits using the equation $p = 9q - 0{\cdot}05q^2$ (Fig. 5) and to the secondary and tertiary responses of rabbit 2/59 (Fig. 6) by the line $p = 6q - 0{\cdot}1q^2$. Sera having titres less than 60,000 when measured by the plaque neutralization method, approximate to a straight line and it is only with the higher titres obtained from sera collected during the tertiary response of one rabbit that the curvilinear relationship between the two becomes evident. It must be stressed that the quadratic function fitted to the points obtained is entirely empirical, and cannot be given any fundamental

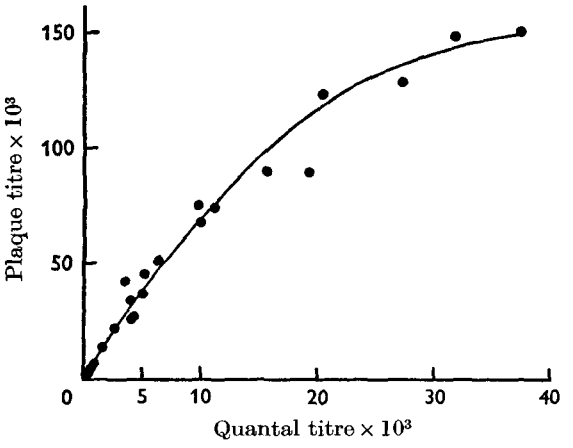

Fig 4

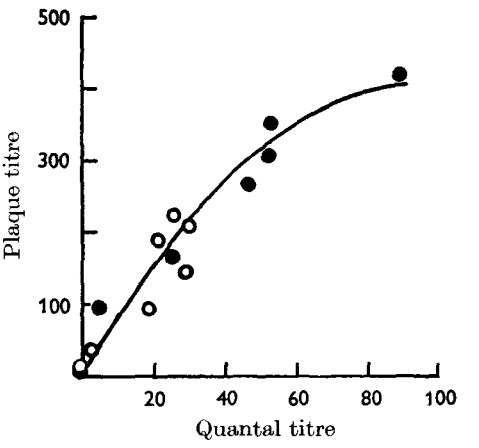

Fig 5

Fig. 4. The relationship between the neutralizing titres determined by the plaque and quantal methods for sera from the secondary and tertiary responses of rabbit 1/59. ——, $p = 8{\cdot}2q - 0{\cdot}12q^2$.

Fig. 5. The relationship between the neutralizing titres determined by the plaque and quantal methods for sera from the primary responses of rabbits 1/59 and 2/59. ●, Sera from rabbit 1/59; ○, sera from rabbit 2/59; ——, $p = 9q - 0{\cdot}05q^2$.

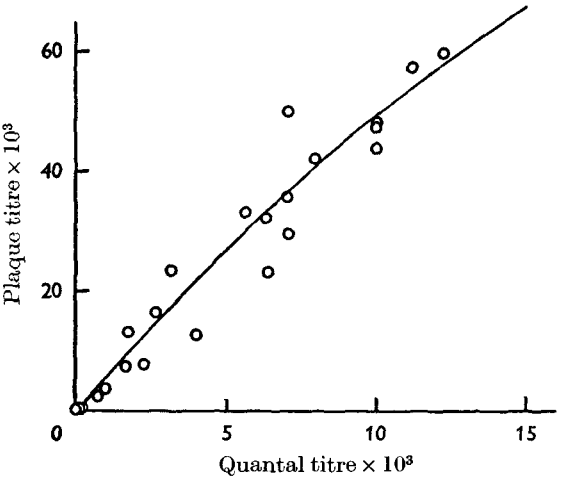

Fig. 6. The relationship between the neutralizing titres determined by the plaque and quantal methods for sera from the secondary and tertiary responses of rabbit 2/59. ——, $p = 6q - 0{\cdot}1q^2$.

significance at this stage; the non-linearity of the correlation between the two antibody estimates is, however, made clear thereby.

The non-linear relationship can be attributed to one of two possibilities:

(1) That the relationship between the absolute antibody concentration and the titre obtained is not linear for one or both tests.

(2) There is a mixture of antibodies in the sera, the proportion of which varies with the state of immunization, each antibody component having a different straight line relationship between absolute antibody concentration and titre when measured by the two tests.

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These two possibilities can be differentiated by titrating a series of dilutions of one of the high titred sera in Fig. 4 by both methods, and plotting the titres so obtained one against the other. If the correlation illustrated in Fig. 4 is a function of the tests themselves, then the titres of the dilutions will follow the same relationship. If, however, it is a reflexion of varying proportions of different antibodies in the sera, then, providing p and q are linear functions of the antibody concentrations, the titres of the dilutions as estimated by the two techniques will lie on a straight line passing through the origin. This will be so because the proportions of different antibodies in a single serum will be constant irrespective of

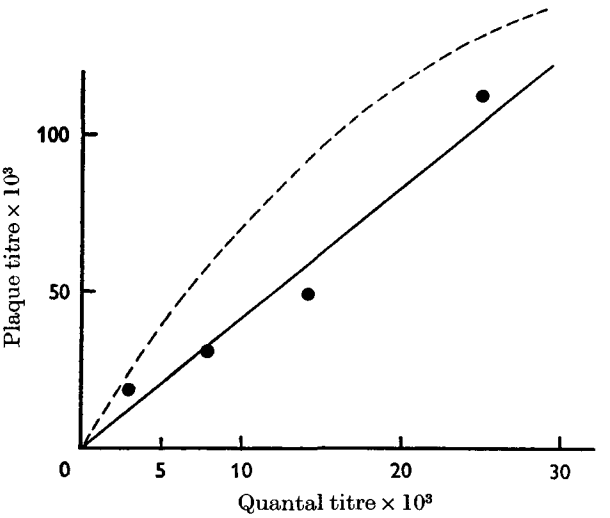

Fig. 7. The relationship between the neutralizing titres determined by the plaque and quantal methods for dilutions of serum 'V' of rabbit 1/59. - - - -, Relationship exhibited in Fig. 4.

dilution. The results of such an experiment (Fig. 7) clearly demonstrate that the two tests are linearly and proportionally related, and that departures from such a relationship are most probably due to varying proportions of different antibodies in the high titred sera.

DISCUSSION

It has been stated that the accuracy of the plaque technique should be far greater than a quantal method using a practicable number of tubes, (Dulbecco, 1955, Lorenz, 1962). In this study the error of the two tests, performed as described here, was very similar and this was due primarily to the large variance of the plaque count at a given dilution. It may be that this could be reduced by removing the inoculum before overlaying, but this, of course, would no longer be the technique accepted at the onset of the study. Cooper (1961) has pointed out that the day-to-day variation in plaque count in the suspended cell method can be reduced by various techniques. These include the use of galactose in place of glucose-carbonate, treating virus with sodium deoxycholate before dilution, and

the inclusion of various supplementary components in the medium. He also advocates the use of tetrazolium salts for the differentiation of plaques in preference to neutral red. However, as has already been demonstrated, it is important to detect plaques as early as possible, and tetrazolium salts can only be used at the time of the final count. It should be added that Cooper (1961) recommends the use of larger plates as the plaque count increases. Even so, considering the area available for the number of plaques present, it is probable, arguing on the basis of this study, that the loss of plaques due to overlaying and coalescence would be of measurable proportions. A further obvious method to reduce the variance of the plaque count at a given virus concentration would be to increase the number of estimates at that concentration, but this would impose severe restrictions on the number of sera that could be assayed at a given time. The relative cost, in time and materials, of these two tests, as employed in this study, is illustrated by the fact that, for every three sera estimated by the plaque technique, 12–15 sera can be titrated by the quantal method. Considering the slight increase in accuracy of the plaque neutralization test over the quantal, in relation to its cost, there seems little justification for the use of the former as a basic routine method. It is obvious, however, that owing to its greater sensitivity, which has also been demonstrated by Ginsberg, Kasymow & Al'tshtein (1960), it has a role to fulfil in the titration of low titred sera.

Comparison of the antibody titres determined by the two methods shows a non-linear relationship despite the absolute antibody concentration and titre being linearly related in both tests. The two sets of values for antibody titres are approximately proportional at titres of less than 60,000 as determined by the plaque method but with increasing stimulation, especially in the tertiary response, the antibody produced differs from the earlier antibody; i.e. there is evidence of inhomogeneity of the antibody produced in response to multiple inoculations of type 1 poliovirus. These findings agree with those of Svehag & Mandel (1964*a*, *b*), who showed that differing proportions of 7*s* and 19*s* antibody are produced in rabbits according to the state of immunization when inoculated with the Brunhilde strain of type 1 poliovirus.

SUMMARY

Sera from two rabbits bled after one, two and three inoculations of concentrated type 1 poliovirus tissue culture fluid were assayed for neutralizing activity by a quantal and a plaque method. The plaque counts in these tests have been shown to approximate to a Poisson distribution as determined by the relationship between the variance and the mean of replicate counts. However, there is a considerable scatter of the points in such a comparison and this accounts for the absence of the expected increase in accuracy of this plaque method over the quantal.

The relationship between the antibody content of the sera determined by the two methods is non-linear. This can be attributed to inhomogeneity of the antibody produced which increases with the number of antigenic stimuli given.

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