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A modified metabolic inhibition test for the titration of poliovirus neutralizing antibodies

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INTRODUCTION

A metabolic inhibition or colour test for the titration of poliovirus neutralizing antibodies was introduced by Salk, Younger & Ward in 1954. Since then, variations in the standard technique have been suggested by other workers (Robertson, Brunner & Syverton 1955; Lipton & Steigman, 1955; Melnick & Opton, 1956). In these tests, dilutions of serum are mixed with a small amount of virus in test tubes or on plastic plates; monkey kidney or HeLa cells, suspended in a suitable medium containing phenol-red and glucose, are added and then liquid paraffin is used for sealing the cultures. These are then incubated for 6–7 days. If the virus is neutralized by the serum, the cells metabolize the glucose, producing acid which turns the phenol-red yellow, but if the virus is not neutralized the cells are destroyed by the virus, the glucose is not metabolized to any extent and the medium remains pink or red. Serum titres can be read by the colours produced at the end of the test.

The modification of the test proposed here has been used in this laboratory for over 2 years and has proved more satisfactory than the standard technique. It will be referred to as the galactose colour test.

METHOD

Media

The following modifications of standard solutions and of lactalbumin medium are used:

Lactalbumin hydrolysate medium:

Lactalbumin hydrolysate, 1 % in Hanks' solution (no glucose)	100·0 ml.
Inhibitor-free calf *or* monkey serum *or* 7 % bovine albumin Fraction V	4·0 ml.
2·5 % glutamine	2·0 ml.
0·5 % valine	0·5 ml.

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10 % glucose	0·05 ml.
4·4 % $NaHCO_3$	4·0 ml.
10 % galactose	2·0 ml.
Penicillin and streptomycin (20,000 units/ml. and 10,000 units/ml)	1·0 ml.
Nystatin (20,000 μg./ml.)	0·5 ml.

Hanks's balanced solution:

Solution A:	NaCl	80 g.
	KCl	4 g.
	$MgSO_4 7H_2O$	2 g.
	Distilled water	2 l.
Solution B:	Na_2HPO_4	0·5 g.
	KH_2PO_4	0·6 g.
	Distilled water	2 l.
Solution C:	$CaCl_2$	1·4 g.
	Distilled water	2 l.

Make up each solution separately and when dissolved mix, make up to 10 l. with distilled water and add 50 ml. of 0·2 % phenol red.

For test use:

Hanks' solution (no glucose)	500 ml.
4·4 % $NaHCO_3$	1·5 ml.
Penicillin and streptomycin	5·0 ml.
Nystatin	2·5 ml.

Phosphate buffered saline and versine solution:

Ethylene-diamine-tetra-acetic acid di-sodium salt (versene)	2·0 g.
NaCl	80·0 g.
KCl	2·0 g.
Na_2HPO_4	11·5 g.
KH_2PO_4	2·0 g.
Distilled water	10·0 l.
Autoclave at 10 lb. for 10 min. If no di-sodium salt is available, use:	
Ethylene-diamine-tetra-acetic acid	2·0 g.
NaOH	0·67 g.

Glucose solution:

Glucose	10·0 g.
Difco yeast extract	0·2 g.
$NaHCO_3$	4·0 ml.
Immune monkey or human serum	10·0 ml.*
Hanks' solution	100·0 ml.

* This can be added as a refinement to stop the later development of virus and eliminates most of the rare intermediate colours which occur with the standard method.

Technique of the galactose colour test

New, unwashed, 2 in. × $\frac{5}{8}$ in. neutral glass test tubes are used routinely for the test. They are sterilized by hot air in metal racks covered with aluminium foil. New, unwashed, soft glass tubes have also been found satisfactory as long as each batch is tested before use to show that its tubes will produce adequate cell cultures. Serum dilutions are prepared in Hanks' solution, 0·25 ml. of each being added to the tubes employed for each dilution. After the addition of 100 $TCID_{50}$ of virus suspension in an equal quantity of this solution, the serum-virus mixtures, together with the usual controls, are incubated for 6 hr. at 37° C. to allow full neutralization of virus to take place. These mixtures can then be left overnight at 4° C., if necessary, before completing the test.

After neutralization, 0·5 ml. of a suspension of secondary monkey kidney cells is added to each tube, followed by 0·75 ml. of liquid paraffin. The tubes are then incubated at 37° C. for 24 hr. in the inclined position and then for 4–5 days vertically.

The secondary monkey kidney cell suspensions are prepared from primary cultures grown in either 20 oz. medical prescription bottles or on all sides of rotating hexagonal Pyrex feeding bottles (Mair & Tobin, 1960). After washing the cultures with 20–30 ml. buffered saline, 30 ml. versene solution is added to each prescription bottle and 20–30 ml. to each feeding bottle. The bottles are then incubated at 37° C. for 15–30 min., the prescription bottles being stationary and the feeding bottles rotated at 12 r.p.h. The bottles are then shaken to remove any cells not released from the glass and the suspension centrifuged at 500 r.p.m. (RCF. 60) for 15 min. The supernatant is discarded and the cells resuspended in lactalbumin hydrolysate medium at a concentration of 150,000 cells per ml. An equally satisfactory procedure, which avoids centrifugation, is the addition of 1 % of a 2 % solution of calcium chloride to the cell suspension to neutralize the versene and then diluting to the required cell concentration with lactalbumin medium.

After the 5–6 days' incubation, 0·2 ml. of glucose solution is added to each tube. The cultures are then incubated for a further 24–48 hr., after which time the colour change will have been completed, those tubes in which virus has been neutralized turning yellow and those in which no neutralization has occurred remaining pink or turning red. The tubes are then discarded.

Automatic pipettes can be used for the addition of virus, cells, paraffin and glucose to the tubes.

EXPERIMENTAL RESULTS

The results of a comparison between the galactose colour test and the cytopathic test have already been published (Perkins & Evans, 1959); the results obtained by both methods were similar for poliovirus antibody.

The galactose colour test has also been applied satisfactorily for testing antibodies of the Coxsackie B group and ECHO 9 viruses. It was used in a study similar to that reported recently by Cook & Smith (1960). Of 48 boys from three public schools near London, bled in July 1958 or March 1959, 15 % had antibodies

to Coxsackie B1 virus, 17% to B2, 19% to B3, 23% to B4, 2% to B5 and 98% to ECHO 9 virus.

Additional information about the precision and reproducibility of the test described in this paper was obtained by carrying out another experiment in which seven sera were tested for poliovirus Types 1 and 3 antibodies at twofold dilutions (ranging from 1/40 to 1/2560) with four tubes per dilution. The whole experiment, using the same sera, was repeated on each of 4 days, and on a further 2 days the difference between consecutive dilutions was widened to become fourfold.

The tubes in which virus was neutralized (yellow tubes) were recorded as negative reaction and the tubes in which no neutralization had occurred (red tubes) were recorded as positive. The results of the first day's experiment with Type 1 are given in Table 1.

Table 1. *Experimental results of tests carried out on one day*

Dilution ...	40	80	160	320	640	1280	2560	50% end-point	Tit
Log scale...	+3	+2	+1	0	−1	−2	−3		
Serum									
a	− − − −	− − − −	− − − −	− − − −	− − − +	+ + + +	+ + + +	−1·33	80
b	− − − −	− − + −	+ + − −	+ + + +	+ + + +	+ + + +	+ + + +	+1·00	16
c	− − − −	− − − −	− − − −	+ + + +	+ + + +	+ + + +	+ + + +	+0·50	22
d	− − − −	− − − −	− − − −	− − − +	− − − +	+ + + +	+ + + +	−1·33	80
e	− − − −	− − + −	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+1·67	10
f	− − − −	− − − −	− − − −	− − − +	+ − − +	+ + + +	+ + + +	−1·00	64
g	− − − −	− − − −	+ + − +	+ + + +	+ + + +	+ + + +	+ + + +	+1·33	12

STATISTICAL ANALYSIS

To simplify the calculations, the dilutions have been transformed to a log scale, as shown in the second line of the table. The 50% end-point was obtained by linear interpolation on this scale and the titre was estimated by transforming this value back into the original units. An analysis of only the Type 1 antibody titrations is given, as the findings with Type 3 were similar.

The 50% end-points were estimated for each serum tested on each day by the method described above (Table 2). From inspection of the table, it is obvious that the method is reproducible from day to day and that the differences in titre for different sera are real differences and not random fluctuations. This can be confirmed by analysis of variance on the end-points for the first 4 days (where the data are complete), which shows that there is no significant difference between days but a highly significant difference between sera ($P < 0{\cdot}001$).

The approximate method for estimating titres, which has been used so far, is quite adequate for the purpose, but there is no satisfactory way of attaching limits of error to those estimates. In order to do this it is necessary to use the standard method of probit analysis (Burn, Finney & Goodwin, 1950), when the percentage responses are transformed to probits and the dilution which produced a 50% response is calculated from the fitted probit line. To distinguish these values

from the approximate ones, they will be denoted by the letter m with standard errors S_m.

A full analysis of the results of each separate test was not possible, for it is necessary to have at least two dilutions which give responses other than 0% or 100% for the method to be used. The information for each serum was therefore combined over the first 4 days and analysed as a single group (Table 3).

Table 2. *50% end-points obtained by repeated testing of the same sera*

Day ...	1	2	3	4	5*	6*	Mean	G.M. titre
Serum								
a	−1·33	−2·33	−1·50	−1·50	−2·00	−1·67	−1·72	1054
b	+1·00	+1·00	+1·00	+0·67	+2·33	+1·67	+1·28	132
c	+0·50	0·00	+0·67	+1·00	+1·00	+0·33	+0·58	214
d	−1·33	−1·00	+0·33	−0·33	−1·67	−1·00	−0·83	569
e	+1·67	+1·00	+2·00	+2·67	+2·00	+2·00	+1·89	86
f	−1·00	0·00	−1·00	−1·33	0·00	0·00	−0·56	472
g	+1·33	+0·50	+1·50	+1·67		+1·50	+1·30	130
Mean	+0·12	−0·12	+0·43	+0·40	+0·28	+0·40	+0·25	269

* Only tested at dilutions −3, −1, +1 and +3 on days 5 and 6.

Table 3. *Number of tubes (out of 16) in which virus was neutralized for each serum*

Log dilution ...	−3	−2	−1	0	1	2	3
Serum							
a	0	3	15	16	16	16	16
b	0	0	0	1	9	15	16
c	0	0	0	2	13	16	16
d	0	1	6	11	16	16	16
e	0	0	0	0	2	9	15
f	0	0	8	13	15	16	16
g	0	0	0	0	6	14	16

The resulting m values (Table 4) were in close agreement with the approximate estimates averaged over each serum (Table 2).

From the standard error it appears that if two sera are being compared (at twofold dilutions with 16 tubes per dilution) the minimum differences, between the resulting titres, which would indicate a difference in antibody level, is about half a dilution. From this, it is possible to deduce that in a single test, using four tubes for each serum dilution, a difference in titre of one dilution could be just significant.

The precision lost by doubling the dilution was estimated from the combined results of all sera for each day (Table 5). The standard errors for days 5 and 6 were twice as large as those for the other days, indicating that the precision was halved. These results are summarized in Table 6.

The significance of the difference in antibody level of two groups of sera is dependent on the variability within a group. From a direct estimate of the variance of one day's test (i.e. a column of Table 2) it is likely that a difference of more than

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$1\frac{1}{2}$ dilutions between the geometric mean titres for this and some other group of seven sera would indicate a significant difference between the groups.

Table 4. *Log titres and their standard errors*

Serum	m	S_m
a	−1·63	0·12
b	0·96	0·15
c	0·56	0·13
d	−0·65	0·17
e	1·88	0·16
f	−0·77	0·17
g	1·25	0·14

Table 5. *Log titres and their standard errors*

Day	m	S_m
1	0·24	0·16
2	−0·18	0·16
3	0·38	0·16
4	0·47	0·19
5	0·28	0·29
6	0·57	0·38

Table 6. *Minimum differences in titre indicating a significant difference between neutralizing antibody levels of single sera*

	Number of tubes at each level	
	4	16
Twofold serum dilution	1 twofold dilution	$\frac{1}{2}$ twofold dilution
Fourfold serum dilution	2 twofold dilutions	1 twofold dilution

DISCUSSION

The advantages of the galactose colour test over the standard techniques in which glucose is present from the beginning are that the variation in cell concentration within certain limits is unimportant, that the time of colour change is under control and that the colour change is more distinct. From 40,000 to 150,000 cells per tube have been used satisfactorily. As would be expected, with the lower cell count the serum titres tend to be a little higher than with the larger number of cells. In the standard test the time of colour change varies as it depends on the number and vitality of the secondary cells and can occur too early, in which case sodium hydroxide has to be added to retard the colour change. Occasionally it never occurs, or so incompletely that reading of the result is difficult. These hazards are eliminated in the galactose colour test.

The addition of human or monkey antibody-containing serum to the glucose solution may improve poor cultures and, because of the presence of poliomyelitis antibodies, may cut off late virus growth, giving a clear yellow or red in almost every tube. Intermediate colours are rare, but if there is any doubt the cell sheet

can be easily examined microscopically as it is on the side and not on the bottom of the tube.

The use of tubes rather than disposable plastic plates is preferred because the serum dilutions can be made more safely in them, they are easier to prepare, sterilize and handle. The cultures can be observed microscopically and the test appears to work somewhat better in them. The absence of glucose was an advantage in two tests which were contaminated, one by a yeast and the other by *Pseudomonas fluorescens*. In one, the cultures could be read microscopically so the test was not invalidated and in the second the addition of polymyxin with the glucose solution inhibited the contaminant so that the normal colour change took place.

Galactose is metabolized very slowly by monkey kidney cells (Dr L. F. Hewitt—personal communication). A trace of glucose is an advantage as the cells grow out and form sheets somewhat more quickly than in a medium containing galactose alone. Galactose can replace glucose for the maintenance of cultures; human amnion and monkey kidney cultures have been maintained in it in good condition for at least three weeks. This action cannot be accounted for by undetectable traces of glucose in the galactose itself. The very low levels of glucose or the presence of galactose have no inhibiting effect on the titres of those enteric viruses tested in monkey kidney cell cultures.

In the enteric virus antibody survey in schoolboys, the ECHO 9 titres were low and presumably indicated infection prior to 1956—a year in which this virus was widespread—and, in fact, no cases suggestive of this infection occurred at any of the schools. At one school, only one of sixteen boys tested had antibody to Coxsackie B3 and no outbreak was noted although many cases had occurred in the neighbourhood the year before the serum samples were taken (Kendall, Cook & Stone, 1960).

SUMMARY

A modification of the metabolic inhibition test for titrating poliomyelitis antibody is described. The method depends on the substitution of galactose for glucose in the initial medium to eliminate colour change during the period of virus activity. Neutralization is detected by the addition of glucose at the end of the period, when the usual colour changes associated with this type of test occur. A statistical analysis of the results obtained is given.

This method can also be used for titrating antibodies to other enteric viruses.

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