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ASSAY OF POLIOMYELITIS NEUTRALIZING ANTIBODY IN DISPOSABLE PLASTIC PANELS *

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SYNOPSIS

A method for titration of poliomyelitis neutralizing antibodies in disposable plastic panels is described. This method is a modification of those reported from the laboratories of Salk and of McLean, in which antibody is measured by protection of monkey-kidney cell suspensions from the metabolic inhibitory influence of the polioviruses.

White polystyrene panels, inexpensive and commercially available, are sterilized by alcohol or ultraviolet light. Only 20,000 to 30,000 passage cells are used per cup. The cell suspension is obtained by treatment of primary kidney cultures with a chelating agent, Versene. Such passage cells have a more uniform metabolic activity than trypsinized cells obtained directly from monkey kidney. Cell suspensions may be stored in the refrigerator for up to three weeks and still prove useful for the colour test. They are standardized for each test by a prior titration of their viability, the results of which are available in less than 24 hours. Both for the primary outgrowth from trypsinized monkey kidney and for the colour test itself, the simple lactalbumin hydrolysate medium is used. Panels are sealed with a paraffin oil of high viscosity.

It is our purpose to describe a method of poliomyelitis neutralizing antibody assay in which oil-covered cups of a white plastic panel are substituted for the more conventional arrangements making use of tube-

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** Who Regional Poliomyelitis Centre for the Americas

supports, culture tubes, and stoppers. Simplified tests for antibody have been developed by a number of investigators in the last few years [1-4, 7-10] and the present one is a modification of those already described. It has proved advantageous in our laboratory where several thousand human sera have been titrated by the technique reported here.

The basis of the colour test for poliovirus or its antibody is found in the observations of Robbins, Enders & Weller [8] on the difference between the pH of virus-infected and normal cultures. A uniformly dispersed suspension of monkey-kidney cells enabled Salk, Youngner & Ward [10] to use phenol red in this cell culture system as an indicator in titrating poliovirus or its antibody. Conditions for employing HeLa cells in the test have been described by Lipton & Steigmann [4] and by Robertson and co-authors.[9] The advantage of serially cavitated plastic for poliomyelitis neutralization tests was first pointed out by Barski & Lépine,[1] who used the microscope to follow the cytopathogenic action of the virus on human embryonic fibroblasts. In this instance, evaporation from the cultures was prevented by adhesive Cellophane tape. The transfer of the metabolic inhibition test to transparent vinyl panels was accomplished by Rightsel et al.[7]

The same basic principle which underlies any virus neutralization test—namely, that antibody specifically neutralizes the infectivity of the virus—also applies to the pH colour antibody test. The colour test employs known quantities of cell suspensions which are inoculated into plastic panel cups one hour after incubation of the serum-virus mixture. The colour test utilizes the fact that, with continued cellular growth in the presence of an immune serum-virus mixture, acid products of metabolism lower the pH of the medium. This effect is readily observed by incorporating the indicator dye, phenol red, into the medium. Phenol red is red at pH 7.4 to 7.8. It becomes salmon and finally yellow as the pH drops to below 7.0. Conversely, cell necrosis, induced by the virus, leaves the medium red, for the dying cultures fail to reach the degree of acidity exhibited by the control cultures. The test can thus be read by colour change alone rather than by the microscopic presence or absence of cellular degeneration.

The modifications of the colour test to be described here include the introduction of (*a*) inexpensive disposable white styrene panels; (*b*) the use of " stabilized " passage cell suspensions, obtained from monkey-kidney cultures by treatment with a chelating agent (Versene); (*c*) the substitution of the simple lactalbumin hydrolysate medium for the more complex media like Connaught No. 199, and (*d*) the " sealing " of the panel cups with a paraffin oil of high viscosity, as recommended by Salk for the tube test.

Materials

1. The *diluent* used for serum and virus is prepared from three stock solutions:

(*a*) Lactalbumin solution, 5%

Lactalbumin enzymatic hydrolysate	25.0 g
Water	500.0 ml

Autoclave 10 minutes at 10 pounds per square inch (0.70 kg per cm^2).

The solution is stable in the refrigerator. If a precipitate forms it can be removed by light centrifugation.

(*b*) Hanks' solution, 10×concentrated

NaCl	80.0 g
KCl	4.0 g
$CaCl_2$	1.4 g
$MgSO_4$, 7 H_2O	1.0 g
$MgCl_2$, 6 H_2O	1.0 g
Na_2HPO_4	0.6 g
KH_2PO_4	0.6 g
Glucose	10.0 g
Phenol red solution, 0.5%	40.0 ml
Water	to make 1 litre

Sterilize by filtration.

(*c*) Phenol red solution, 0.5%

Phenol red	5.0 g
NaOH, 0.1 N	150.0 ml
Water	to make 1 litre

The stock solutions and antibiotics are combined as follows:

Hanks', 10×concentrated	500.0 ml
Lactalbumin solution, 5%	500.0 ml
Sterile water	4000.0 ml
$NaHCO_3$, sterile, 7.5%	35.0 ml
Tetracyclin solution, 10 mg/ml	12.5 ml
Penicillin, 100,000 units/ml	10.0 ml
Streptomycin, 320 mg/ml	2.5 ml
Nystatin, 500,000 units/ml	1.0 ml

2. The *nutrient* medium, used for cell suspensions and cell titrations, is prepared by adding 2.0% calf serum to the diluent described above.

3. Versene [a] solution, for use in obtaining suspensions of passage cells, is prepared by combining:

Versene	0.2 g
NaCl	8.0 g
KCl	0.2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g
Water	to make 1 litre

Sterilize by autoclaving.

4. The dye-fixative mixture used for routine cell counts is 0.1% crystal violet in 0.1 M aqueous citric acid. This mixture does not distinguish between living and dead cells. For vital staining a 0.01% solution of neutral red is used. Dead cells are identified with the aid of 1.0% trypan blue solution.

5. Paraffin oil [b] is used to "seal" the panel cups. While the oil may be sterile as packaged, it should be autoclaved at 20 pounds as a matter of routine.

6. The Cornwall continuous pipetting unit [c] serves to distribute diluent, virus suspensions, cell suspensions, and oil in all replicate operations (see Fig. 1). A 4-inch, No. 13 (102 mm×2.40 mm), hypodermic needle, whose point has been cut off, makes a suitable cannula, especially when bent to a slight curvature. For oil, a specially large cannula is recommended.[d]

7. A cell dispenser may be made by making four symmetrically placed indentations about the bottom of a 1-litre Erlenmeyer flask and adding two ports through which the cell suspension is to pass to two Cornwall units for simultaneous addition of cells to two panels. The indentations, which increase mixing efficiency, are like those of the automatic trypsinization assembly devised by Rappaport.[e] The ports are introduced at the side of the flask as near to the bottom as is practicable so that the cell suspension may be drained without waste. Adequate mixing of cell suspensions, the preparation of which is explained in detail later, is ensured by using a magnetic mixing assembly.

8. Nine-inch (about 23 cm) lengths of household aluminium foil from rolls 12 inches (about 30 cm) wide are autoclaved. These sterile foil "covers"

[a] Di-sodium salt of ethylenediamine-tetra-acetic acid. Obtainable from Versenes, Inc., Framingham, Mass., USA.

[b] Drakeol, No. 35, Pennsylvania Refining Co., Butler, Pa, USA.

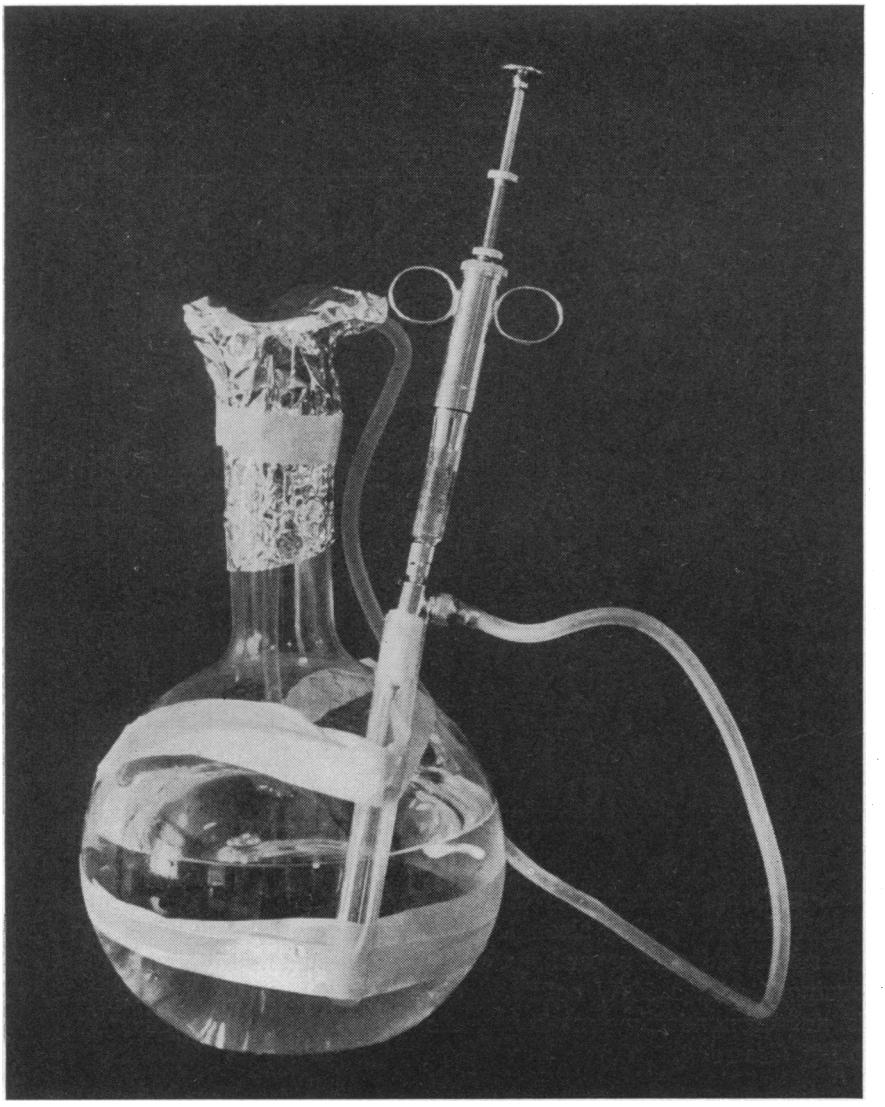
[c] No. 1251, Becton, Dickinson & Co., Rutherford, N. J., USA.

[d] Luer-lok portion of the pipetting unit's valve can be modified as an oil cannula for Cornwall unit. Rathgeber Laboratories, 226 County St., New Haven, Conn., USA.

[e] See the article by C. Rappaport on page 147 of this number of the *Bulletin*.

are used to protect filled cups against airborne contaminants. Furthermore, when panels are covered, it is possible to stack them without wetting the bottom of one with the content of the panel below.

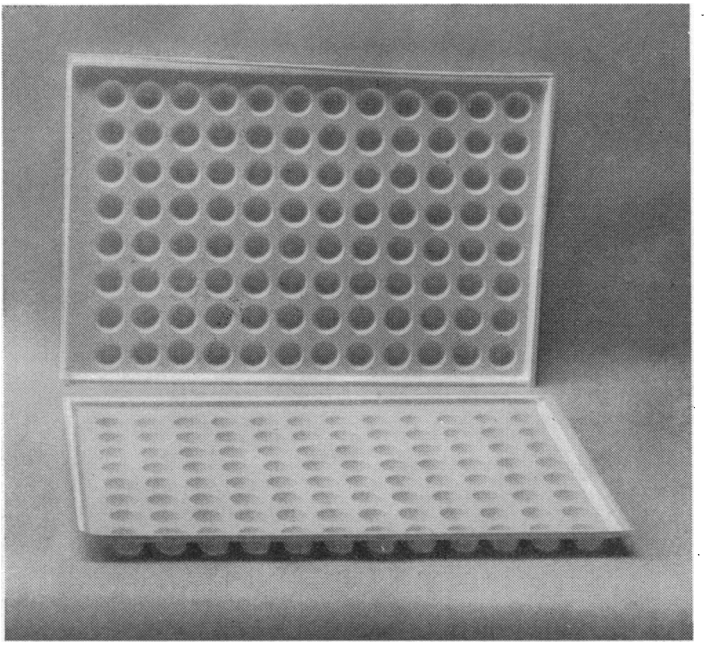
FIG. 1. REPLICATING ASSEMBLY



This convenient assembly, employing the Cornwall continuous pipetting unit, is used for distributing replicate amounts of diluent, virus, and oil. A modified flask described in the text is used in dispensing cells.

9. Panels[a] are moulded from white styrene sheets, 0.5 mm in thickness (see Fig. 2). They contain 96 cups in 12 rows of 8 each; each cup holds a volume of about 2.7 ml. All readings are made by colour. Transparent panels of vinyl plastic have also been used, but the white background of the styrene panel has facilitated reading the colour changes. The styrene plastic is sufficiently rigid so that the panels may be stacked in a small incubator without additional holding racks being required.

FIG. 2. STYRENE PANELS



Panels are prepared for use by successive rinsings in tap water, distilled water, and 70% alcohol. If not used immediately when dry, they are stored in sterile paper bags, or they are nested and placed in a cabinet equipped with a 30-W cold cathode ultraviolet sterilamp.[b] Since re-use is not economically practicable, the panels are steam-sterilized before being discarded.

[a] Linbro Chemical Co., 681 Dixwell Ave., New Haven, Conn., USA.

[b] Irradiation at 18 inches (45 cm) for 60 hours produced no cell-inhibitory changes in panels manufactured by Linbro Chemical Co. from Dow Chemical Co. No. 475 Hi-impact styrene, or from Bakelite Co. VU 1310 clear vinyl plastic.

Methods

Cell suspensions: preparation and handling

Eighty ml of primary trypsinized cells [a] containing 200 000 cells per ml of nutrient medium are placed in a 1-litre Roux bottle and maintained for 5 to 9 days at 37°C. By this time a luxuriant epithelial cell sheet should form and the pH of the medium drop to 7.0 or below. To harvest the cells the nutrient is first thoroughly drained from the bottle. Then 25 ml of Versene solution at 37°C are added, and the bottle is re-stoppered and returned to the incubator. If the bottle has not cooled appreciably, in 20 minutes the cells may be loosened from the glass by two quick shakes of the bottle in a horizontal plane, and poured into a storage bottle. The time necessary for the action of the Versene solution may extend to 40 minutes if the temperature is allowed to fall.

The harvested cells, numbering approximately 20 million per bottle, are prepared for storage at 4°C by adding 75 ml of the nutrient per harvested bottle. The addition of nutrient binds any excess of chelating agent, and centrifugation is unnecessary.[b]

Calculation for further dilution of the cell suspension is based on the following rapid counting procedure: 1 ml of the cell suspension is added to and gently mixed with 2 ml of the crystal violet solution. The haemocytometer chamber is charged with this mixture and the number of cells per ml computed. Versene-treated cells do not clump to the same degree as those from primary trypsinized cultures.

For special purposes, neutral red or trypan blue is used in conjunction with cell counts.

Neutral red: To 1 ml of cell suspension, 0.05 ml of the neutral red solution is added and the mixture is placed at 37° C for 20 minutes. An aliquot is then transferred to the haemocytometer chamber. The metabolically active cell is reflected in what appears to be an accumulation of the red dye in the nucleus. The examination requires good illumination, and a green filter of proper transmission is helpful.

Trypan blue : To 1 ml of cell suspension is mixed 0.05 ml of the trypan blue solution, and the count is done immediately. Owing to the toxicity of the dye itself, delay in charging the haemocytometer chamber and in counting results in inflated totals.

If the cell suspension is not immediately used, the counting is deferred to some 24 hours prior to its anticipated use or is repeated at this time. The

[a] See the article by C. Rappaport on page 147 of this number of the *Bulletin*.

[b] For certain purposes (storage in small volumes, shipping of cells, etc.), the Versene harvests of several bottles may be pooled. The pooled harvests are spun at 600 revolutions per minute for 10 minutes, the supernates are discarded, and the cells are re-suspended in nutrient at a concentration of $1\text{-}2 \times 10^6$ cells per ml.

cell titration and sterility test now to be described should also be performed. Foreknowledge of the sterility of the cell suspension is desirable, and advance incubation of a sample of the suspension at 37°C serves as a safeguard against the hazard of mass contamination originating in the cell suspension. A cell titration in advance of the test measures the physiological activity of the cells. It is performed at several twofold dilutions (like the cell titration control, see below) using 4 stoppered tubes, 13 mm × 100 mm, for replicate incubation of 1.0 ml of each dilution. An index to the condition of the cells is provided by three sorts of observations; (*a*) the colour of the tube cultures (not strictly comparable to a cell titration control performed in a panel because in this instance the stopper traps all the CO_2 produced); (*b*) the morphology of the cells [a] (especially using granulation as an index of morbidity); and (*c*) the condition of the culture as a whole, particularly whether a true sheet or islets of cells are present. After freshly-harvested cells are incubated 16 hours at 37°C, microscopic examination should show complete sheets with a seeding concentration of 135 000-180 000 cells per ml, almost continuous sheets at concentrations of 115 000-150 000 cells per ml, and large islets down to concentrations as low as 95 000 cells per ml. Concentrations above 135 000 cells per ml will cause enough acid to form to bring about a shift from red to yellow. The lesser concentrations mentioned will bring about a change to shades of salmon within the same period. In general 20 000 cells per cup are used within 3 days of their harvest, and up to 30 000 if the suspension is stored from 7 to 10 days.

Test sequence

A convenient volume, about 2.0 ml, of the lowest serum dilution to be tested is heated for 30 minutes at 56°C. This volume, usually of a 1:4 dilution, provides enough material for test at this dilution and also for the preparation of further serial dilutions of heat-inactivated stock. In the correspondingly located cups of 4 panels are placed $^2/_{10}$-ml aliquots of each serum dilution, according to a planned protocol. Four equivalent panels are so obtained, one of which serves as a control of serum toxicity, while the other three are to be used for challenge of the sera with poliovirus of types 1, 2, and 3. To indicate subsequent treatment, the labels attached to the individual panels bear the symbols, C, 1, 2, or 3, as prefixes to further identification numbering. " C " indicates a control panel, while a numbered prefix refers to the virus type to be added later.

Two serum dilution schedules have proved especially useful. The first, for screening, uses duplicate cups for the testing of sera at 3 dilutions, to wit, 1:10, 1:50, and 1:250. The second, greater in range, uses a single cup to test sera at 1:4, 1:8, 1:16, 1:64, 1:256, and 1:1024 dilutions. Both these

[a] On incubation, the cells settle and adhere to the curved bottom of the test-tube. By lowering the condenser on some models of microscopes, a satisfactory examination of the condition of the cells may be made by centring the bottom of the inverted culture tube in the hole of the stage.

arrangements require a total of 24 cups (6 per panel) for the examination of each serum. Each group of 4 panels provides facilities for testing 16 sera.

When the serum dilutions have been distributed to a quartet of panels, 0.8 ml of the paraffin oil is added to each cup, and a foil cover is placed on each. If desired, the serum-filled panels may be stored for a week in the refrigerator, or even longer in the freezer. Refrigerated panels must be brought to room temperature to decrease the viscosity of the oil before addition of materials is attempted. If the test is completed on the day the serum dilutions are made, the oil may be placed in each cup after the virus or cells have been added.

The virus challenges, 0.2 ml per cup, are added to the serum dilutions in the panels in accordance with the prefix notation of their labels, i.e., type 1 virus is added to panels whose identity label bears the prefix number 1, etc. Each cup should receive 100 TCD_{50}. The panels reserved for serum toxicity control receive a blank of 0.2 ml diluent per cup. These additions are followed by an incubation period of 1 hour at room temperature. All are covered with foil during the period of incubation.

When the hour is past, 0.2 ml of cell suspension is added, and the panels are re-covered with foil and placed in an incubator for 5 or 6 days at 37°C.

In addition to the tests for possible toxicity of individual sera, three additional types of controls are needed:

1. The preparation of virus suspensions containing the desired number of tissue-culture doses presupposes a knowledge of the virus titres. Even so, each test includes a titration of the virus suspension used for challenge of the sera, to provide the specific information as to how nearly the test challenge corresponds to the 100 TCD_{50} intended. If any lot of virus shows a consistent fall in titre on repeated titrations, it is discarded, for the accumulation of non-infectious virus tends to bind antibody, thus lowering serum titres as measured in this test.

Each virus titration is done to include a range of dilutions sufficient to demonstrate the end-points, from a dilution at which all cultures become infected to one at which none is infected. Thus, if 100 TCD_{50} are anticipated in 0.2 ml of the virus suspension when at 10^4 dilution, the titration is extended, step by step, to include the 10^7 dilution, which is ten times greater than that in which the 50% end-point is expected. For $\log_{10}$ intervals, 8 cups are used at each dilution; for 0.5 $\log_{10}$ intervals, 4 cups suffice at each dilution.

Virus stocks prepared in tissue culture are stored frozen at −20°C in aliquots of 1.0 ml in stoppered 13 mm × 100 mm culture tubes. For each test, a separate aliquot is thawed. Stocks are not re-frozen.

2. To compare neutralizing antibody titres achieved in one test with those obtained in another, each test must include a reference serum for

each antibody type. Human serum pools, type-specific monkey hyperimmune sera, and commercial gamma-globulin have been useful. Reference sera are tested at twofold dilutions using 4 cups per dilution, and 50% end-point titres are calculated for each type.

The storage of aliquot tubes of reference sera is recommended. Human sera keep satisfactorily at −20°C at 1:4 and 1:8 dilutions for more than 6 months. Reconstituted, lyophilized monkey hyperimmune serum can similarly be stored at 1:100 dilution.

3. Titration of the cell suspension demonstrates that the cells as used are at proper concentration and possess sufficient viability to produce enough acid to change the indicator from red to yellow during the period of incubation. The cell titration is repeated at the beginning and at the end of the test. At each time 2 ml of the suspension are diluted with 4 ml of nutrient. These 1:3 dilutions, equivalent to the final cell concentration used in virus-serum mixture, are then delivered in 0.6 ml portions to a row of several cups. A second and third row are similarly arranged to contain further twofold and fourfold dilutions, respectively, of these 1:3 dilutions. The cups are covered with paraffin oil and foil and the panel is placed in the incubator with the others. At the time of reading, about 5 days later, the first row should be a bright yellow, the second row a yellow or yellow-salmon, and the third row red.

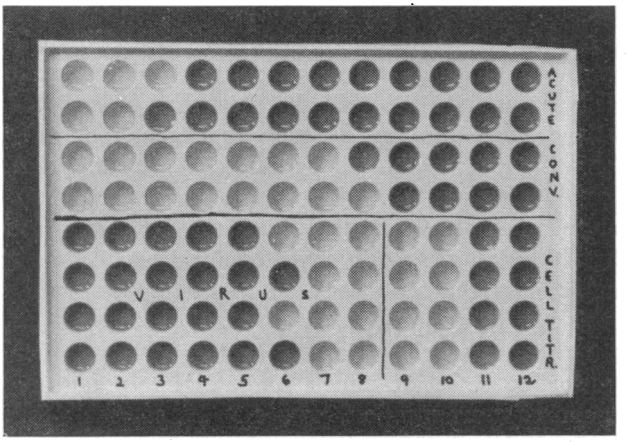
Reading and interpretation

The results are recorded on protocol sheets bearing the imprint of four ruled lattice patterns, each representing the arrangement of cups in a panel (see Fig. 3). A marginal notation on one of the four patterns of a protocol page records both the identity and dilution schedule of the sera whose test results are recorded on that page.

The shift in colour in the cups in which virus is absent or neutralized is expected on the 5th day, but with cells of lessened viability, a few more days may be needed. While end-points will not change with extended incubation, when a panel is repeatedly inspected during the incubation period it can sometimes be observed that a cup which gives a hint of change —say, to a salmon tint—will, a day later, have reverted to red. This seemingly false start towards yellow and subsequent return to a shade close to the original red is probably a reflection of the carbon dioxide passage through the oil. The gradual, and tardy, release of trapped carbon dioxide from cultures occurs when the amount of virus is insufficient to destroy the cells until after the virus has multiplied appreciably. Thus there is sufficient time in such cultures for cells to metabolize sufficiently to produce a colour shift.

If the amount of cells used is excessive, enough non-volatile acid may accumulate to shift the colour from red to yellow before the virus has

FIG. 3. SAMPLE PANEL AT TIME OF READING



This panel illustrates a cell titration, a virus titration, and titrations of acute and convalescent sera from a poliomyelitis patient. Yellow cups, which photograph light, indicate the presence of live, metabolizing cells ; red cups, which photograph dark, in the cell titration indicate that too few cells were added to increase the acidity of the medium significantly. Where virus was added, red cups indicate that the cells have been killed.

Bottom right. The cell titration was carried out using 4 cups per dilution, starting with 25 000 cells per cup in row 9, and with falling twofold dilutions in rows 10, 11, and 12. Row 9, yellow ; row 10, yellow ; row 11, salmon-red ; row 12, red. All cups used in the virus and serum titrations contained 25 000 cells.

Bottom left. The type 1 virus titration was carried out using 4 cups per dilution starting with a concentration of tissue-culture fluid of $10^{-3.5}$ in row 1, and with falling 0.5 log dilutions through row 8. Rows 1 to 5, red ; row 6, two red cups, two yellow cups ; rows 7 and 8, yellow. The 50% end-point is at $10^{-6.5}$. Virus at a concentration of $10^{-4.0}$ (100 TCD_{50}) was added to all cups in the serum titrations.

Top half. The titrations of the sera for type 1 antibodies were carried out using twofold dilutions, starting in row 1 with a dilution of 1: 4. Two cups were used for each dilution. *Acute serum* : Row 1, yellow; row 2, yellow; row 3, one yellow, one red; rows 4 to 12, red. Titre is 1: 16. *Convalescent serum*: Rows 1 to 7, yellow; row 8, one yellow, one salmon; rows 9 to 12, red. Titre is 1: 512.

multiplied sufficiently to kill the cells, and a false interpretation might ensue. When this is suspected (as it might be when an entire virus-challenged panel turns yellow), it is possible to check on the hypothesis by adding base to each cup in order to change the colour from a clear yellow to light salmon. The panels are then returned to the incubator for another 1 to 2 days, when those cups with living cells will have again turned yellow, while the light salmon colour will be retained by those cups whose cells were already dead when the base was added. One drop of 0.05 N NaOH from a non-bevelled 19-gauge (1.10 mm) needle is sufficient to achieve a proper colour difference; more may kill cells.

Colours intermediate between red and yellow are sometimes encountered in cultures which have reached their end-point reactions. That the sera themselves can influence the shades of colour attained is illustrated in serum toxicity control panels. The dilution of a toxic factor is easily recognizable when a series of colour changes from red to yellow is observed

as one progresses to the higher dilutions of serum. The corresponding phenomena, in a virus-challenged panel, may be illustrated by the following observations:

Six cups containing serum dilutions of 1:4, 1:8, 1:16, 1:64, 1:256, and 1:1024 show the colours respectively, red, red, salmon, yellow, yellow, and red. This series indicates that the neutralizing power of the serum is masked at the lower dilutions by some toxic material, whose lessening concentration with further dilution permits cell growth. The renewal of cell inhibition on reaching the 1:1024 dilution is attributed to the insufficiency of neutralizing antibody at that dilution. The end-point is recorded as the highest dilution of serum which neutralizes the challenge dose: in this example it is that contained in the yellow-coloured culture (at 1:256) and this is regarded as the end-point.

The fact that definitive end-points do not change on further incubation makes it possible to set aside panels for later interpretation. Since the incubator encourages evaporation, panels to be set aside for reading at a later time should be placed at room temperature or in the refrigerator.

Results

Viability of stored passage cells

Data on the survival of passage cells stored at 4°C are shown in Table I for three replicate suspensions over a period of 30 days. At weekly intervals,

TABLE I. DIFFERENTIAL COUNTS OF 3 REPLICATE PASSAGE CELL SUSPENSIONS STORED AT 4°C (CELL COUNTS GIVEN AS 1000 CELLS PER ML)

Suspension No.	Method of count *	Days of storage				
		0	7	14	21	30
1	NR	89	98	76	70	18
	TB	18	36	58	73	73
	NR+TB	107	134	134	143	91
	CV	137	165	157	139	133
2	NR	86	70	61	26	4
	TB	23	42	68	105	96
	NR+TB	109	112	129	131	100
	CV	128	135	110	109	67
3	NR	90	79	79	63	17
	TB	18	40	58	51	88
	NR+TB	108	119	137	114	105
	CV	114	165	150	140	94

* NR = neutral red ; TB = trypan blue ; CV = crystal violet. NR plus TB counts were obtained by adding the separate counts for each stain.

three separate counts of these suspensions were made, using, respectively, neutral red solution, trypan blue solution, and the crystal violet mixture, to identify the living, the dead, and the total number of cells. While the count of viable cells (neutral red staining) falls off on storage, it does so only slowly over a period of 21 days. Cells should be used for test purposes during this period but not thereafter.

Protocol of a sample test

Table II presents results of a test which demonstrate the controls that must be set up in each test to make possible its proper evaluation. Readings were made on the 5th day after the addition of cells. Part 1 of the table demonstrates that cups containing 20 000 cells metabolized strongly, for they turned yellow; those containing 10 000 cells still showed demonstrable metabolism, turning salmon. (If a cell concentration which metabolizes to a degree just sufficient to turn salmon in a panel cup is placed in a stoppered tube in which no CO_2 is lost, then the CO_2 accumulation is enough to turn the indicator yellow.) The number of cells placed in all other cups in the test, shown in Parts 2 to 5, was 20 000.

Part 2 demonstrates the virus titration; 50% end-points are determined by the widely used methods of Reed & Muench and of Kaerber.[a] Virus titrations are carried out at the time of each test. The amount used in a particular test, however, is based on the accumulated values of a number of previous titrations, recently performed.

Part 3 illustrates the titration of a serum control. In each test, a serum of known antibody titre against each of the three types must be included. A single serum containing the three types of antibody—or several sera, each containing only one or two antibody types—may be used. The 50% end-point of each serum is determined, as illustrated, using two fold dilutions of serum and 4 cups per dilution.

Part 4 illustrates the results obtained in this test with acute and convalescent sera of a patient from whom type 1 poliovirus was isolated. The interpretation of the serum titrations is shown in Part 5. A rise of type 1 antibodies is obvious; antibodies were present for type 2, but did not rise; antibodies were below the demonstrable level for type 3 in both samples. It should be pointed out that in many poliomyelitis patients, perhaps 50%, antibodies against the infecting type of virus are present at high levels on admission to a hospital, and these antibodies may not change significantly during the remaining course of the illness. In such instances, laboratory confirmation of diagnosis is dependent upon isolation of virus from faecal specimens and complement-fixing antibody determinations.

[a] Detailed examples of the application of both these methods for determining 50% end-points to poliomyelitis virus and antibody titrations may be found in Paul & Melnick.[4]

TABLE II. PROTOCOL OF COLOUR TEST USING VERSENATED CELL SUSPENSIONS

Part 1. Cell Titration

Cell concentrations	Colour * in cup no.			
	1	2	3	4
4 cups containing 20 000 cells	Y	Y	Y	Y
4 cups containing 10 000 cells	S	S	S	S
4 cups containing 5 000 cells	R	R	R	R

*** Y = yellow ; S = salmon ; R = red**

Part 2. Virus Titration

Virus dilutions	Type 1	Type 2	Type 3
$10^{-4.0}$	4/4 *	4/4	4/4
$10^{-4.5}$	4/4	4/4	4/4
$10^{-5.0}$	4/4	4/4	4/4
$10^{-5.5}$	4/4	4/4	4/4
$10^{-6.0}$	3/4	3/4	4/4
$10^{-6.5}$	1/4	0/4	1/4
$10^{-7.0}$	0/4	0/4	0/4

* 4/4 = $\frac{\text{Number of cups showing virus effect (red)}}{\text{Number of cups used}}$

Part 3. Standard Immune Sera Control with Known-Type Monkey Immune Serum plus Test Dose of Virus of Same Type plus Cells.*

Serum dilutions	Colour in cup no.			
	1	2	3	4
0.2 ml of 1:25 serum+0.2 ml diluent+0.2 ml cells (control)	Y	Y	Y	Y
0.2 ml of 1:25 serum+0.2 ml virus +0.2 ml cells	Y	Y	Y	Y
0.2 ml of 1:100 serum+0.2 ml virus +0.2 ml cells	Y	Y	Y	Y
0.2 ml of 1:200 serum+0.2 ml virus +0.2 ml cells	Y	Y	Y	Y
0.2 ml of 1:400 serum+0.2 ml virus +0.2 ml cells	Y	Y	Y	Y
0.2 ml of 1:800 serum+0.2 ml virus +0.2 ml cells	Y	S	R	R
0.2 ml of 1:1600 serum+0.2 ml virus +0.2 ml cells	R	R	R	R

*** Only data for type 1 are illustrated in this part.**

TABLE II. PROTOCOL OF COLOUR TEST USING VERSENATED CELL SUSPENSIONS
(continued)

Part 4. pH Neutralization Test with Sera of Patient Infected with Type 1 Poliomyelitis Virus

Serum dilutions	Type 1 virus *		Type 2 virus *		Type 3 virus *	
	acute	convalescent	acute	convalescent	acute	convalescent
1:4	Y	Y	Y	Y	R	R
1:8	Y	Y	Y	Y	R	R
1:16	R	Y	Y	Y	R	R
1:32	R	Y	Y	Y	R	R
1:64	R	Y	R	R	R	R
1:128	R	Y	R	R	R	R
1:256	R	S	R	R	R	R
1:512	R	R	R	R	R	R
1:1024	R	R	R	R	R	R

* 100 TCD_{50} of each virus used in test. Control cups containing serum dilutions without virus were all yellow.

Part 5. Results of Serum Titres

Type 1 specific monkey serum	1:800
Patient's serum, type 1, acute	1:8
convalescent	1:256
type 2, acute	1:32
convalescent	1:32
type 3, acute	<1:4
convalescent	<1:4

Comparative efficiency of primary and passage cells in tubes and in panels

The difference in potency of passage cells and primary cells in this system is emphasized in Table III, which also furnishes data to show the correspondence of virus and antibody results obtained when materials are tested simultaneously by the tube [10] and panel methods.

Consistency of results obtained by the panel neutralization test

In addition to the comparative results of tests carried out in panels and tubes, the results of a series of consecutive determinations in panels

TABLE III. COMPARATIVE VIRUS AND SERUM TITRES USING TUBE AND PANEL METHODS

	Virus (log of titre)			Immune monkey sera *			Human serum pool H **			Human serum pool L**		
	type 1	type 2	type 3	type 1	type 2	type 3	type 1	type 2	type 3	type 1	type 2	type 3
Original cells †												
Tubes	5.9	5.6	5.1	2500	5000	25 700	630	89	112			
Panels	6.0	5.4	5.8	3070	3540	23 900	615	130	106			
Tubes	6.1	5.6	5.5	1410	2400	16 000	1024	128	150	38	75	89
Panels	6.3	5.3	5.6	2820	8000	22 400	2048	256	212	53	64	75
Tubes	5.9	5.2	5.2	1190	3350	13 300	595	45	32	45	64	45
Panels	5.4	5.5	5.2	4730	4730	16 000	707	150	150			
Passage cells †												
Tubes	5.3	5.5	6.3	1540	1540	7 200	505	128	178	13	27	22
Panels	5.6	5.4	6.0	NT	6100	NT	256	89	64	19	>32	27
Tubes	6.0	5.2	5.2				355	71	141			
Panels	6.0	5.2	5.2	1410	4000	22 000	707	178	89	22	128	32
Panels	5.8	5.2	5.3	2390	2820	13 300	707	106	89	19	38	32
Panels	5.3	4.9	4.9	2820	2820	22 400	1680	NT	178	22	45	45
Panels	5.3	5.2	5.0	3350	3350	32 000	1410	89	178	45	89	64
Panels	5.9	5.5	5.2	NT	2820	16 000	1410	150	212	16	106	53
Panels	5.5	5.1	5.0	4730	4000	NT	1410	212	NT	45	89	NT
Panels	5.9	4.8	5.0	4460	3350	28 200	890	178	178	35	89	45

NT = Not titrated

* Standard-type specific antisera prepared by Dr H. A. Wenner for the National Foundation for Infantile Paralysis, USA.

** Standard-type " high "- and " low "- titre human serum pools used in the 1954 Poliomyelitis Vaccine Evaluation Program in the USA.

† Original trypsinized cells used at concentration of 300 000 cells per tube ; 150 000 cells per panel cup ; Versene-treated passage cells at 25 000 cells per tube or panel cup.

are also listed in Table III. The degree of variation is no greater than that found in tube tests. As is to be expected, the degree of accuracy and reproducibility is increased with the number of panel cups used at each serum dilution. The data in Table III were obtained using twofold dilutions of serum with 4 cups for each type at each dilution.

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RÉSUMÉ

Les auteurs décrivent une variante de la méthode de titration colorimétrique des anticorps antipoliomyélitiques neutralisants, dans laquelle on emploie un plateau de plastique creusé de cupules, remplaçant éprouvettes, bouchons et porte-tubes. Cette technique a déjà fait ses preuves dans le titrage de plusieurs milliers de sérums.

Des quantités connues de suspensions cellulaires sont déposées dans les cupules de plastique après une heure d'incubation du mélange immunsérum-virus. Le principe du test est le changement de pH, mis en évidence par un indicateur, en l'occurrence le rouge de phénol, qui, rouge à pH 7,4-7,8 devient saumon, puis jaune à pH 7,0. La croissance cellulaire qui se poursuit en présence du mélange immunsérum-virus, met en liberté des acides qui abaissent le pH du milieu et font virer le rouge de phénol au jaune. La nécrose cellulaire provoquée par le virus non neutralisé, au contraire, maintient l'indicateur au rouge, la culture en voie de destruction ne produisant pas le degré d'acidité de la culture témoin.

La modification du test colorimétrique que préconisent les auteurs comprend: *a*) l'utilisation de plateaux de polystène peu coûteux; *b*) celle de suspensions cellulaires «stabilisées», provenant de cultures de cellules rénales de singe traitées par un agent assurant la mise en suspension des cellules (tel le Versene); *c*) celle d'un milieu à base d'hydrolysat de lactalbumine remplaçant le milieu N° 199; *d*) celle d'huile de paraffine à haute viscosité pour recouvrir les cupules.

Après avoir décrit en détail le matériel nécessaire et la technique de la méthode, les auteurs la comparent avec la méthode usuelle de neutralisation en tubes. Les résultats indiquent que le degré de variation n'est pas plus élevé avec la méthode des cupules et que, comme on pouvait s'y attendre, le degré de précision et de reproductibilité augmente avec le nombre de cupules utilisées pour une même dilution de sérum.

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