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REPRINTED ARTICLE

For commemoration of 50th anniversary of foundation of this journal, an article of historical interest, and of general importance is reprinted in the present volume accompanied by current editorial notes.

On 6 June 1955 an article was submitted for publication by Gyula Takátsy M.D., senior virologist at State Institute of Public Health, Budapest, initiating a new era – the era of microtechniques – in microbiology and other fields of natural science. The article with reference 1955, 3: 191–202 together with current editorial notes appear below.

THE USE OF SPIRAL LOOPS IN SEROLOGICAL AND VIROLOGICAL MICRO-METHODS

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The classical method of serological titration, that described by WIDAL, involves the use of pipettes and tubes for making serial dilutions. In virus research, where serological titration is a common procedure, the classical method is gradually losing ground in favour of techniques in which dilutions are made in grooves of plates made of glass or of plastic material. Such techniques have been described by NARTSISSOV [1], SALK [2], as well as by VAN DER VEEN and MULDER [3]. The advantage of the plate technique is that it facilitates supervision of parallel reactions and that plates are considerably easier to handle than tubes. The technique has been found particulary useful in virus research, since it is not negligible how much has to used from costly immune sera and antigens.

FULTON and DUMBELL [4] have developed a complement fixation test in which drops of reagents are brought together on a plain plastic plate. A drawback of the method is, however, that it cannot be used in other types of serological test and that serial dilutions must be prepared separately in tubes and transferred therefrom onto the plate.

The favourable experience obtained with my own serological micro method [5] over a period of years has induced me to present a detailed description of its technique.

The essence of the method is that serial dilutions are made in micro volumes, instead of by pipettes and tubes, by means of a calibrated spiral loop in the grooves of plastic plates. The diluent and the reagents are measured in by means of a calibrated instillator.

Equipment*

Spiral loop. This is made of heatresistant, elastic wire and is so formed that the volume of fluid taken up is determined almost exclusively by the space surrounded on every side by spiral wire. This is possible since the coiling is rather dense and thus the fluid taken up has no free surface of such a size which would possess a surface tension capable of influencing the volume of fluid. The loops (Fig. 1) take up 0.025, 0.05, 0.1 and 0.2 ml of fluid, respectively, with a maximum error of ±2 per cent. The loops are provided with a light metal holder.

Plates. These are made of translucent plastic, measure 8 × 68 × 130, or 8 × 95 × 130 mm in size and contain 6 × 12 or 8 × 12 grooves, 6 mm in diameter each. The grooves have a funnel shaped bottom and can take up 0.15 ml fluid.

Instillator. This is a small rost-free metal tube enclosed in plastic and its tip is so formed that the single drops of any fluid which has the same or nearly the same surface tension and specific gravity as those of water (physiological salt solutions, diluted solutions of proteins and, in general, aqueous solutions not containing an organic solvent), are 0.025 ml in volume. The instillator is provided with a cone by means of which it can be attached to the end of an adequately drawn-out ungraded pipette (Fig. 2).

The essence of the spiral loop dilution method. The diluent is measured in the grooves by means of the instillator. The single volumes are equal with, or 2, 3 etc. times that of the loop, depending on the steps of the dilution series to be prepared (1 : 2, 1 : 3, 1 : 4 etc.). With the flamed and cooled loop the surface of the fluid is touched, on which the fluid fills the space enclosed by the spiral. Surface tension will keep the fluid taken up in the loop. The loop filled with fluid is dipped into the first groove and rotated therein determinedly, causing the fluid introduced

* All the equipment described may be purchased from Orvosi Műszer KV. Budapest.

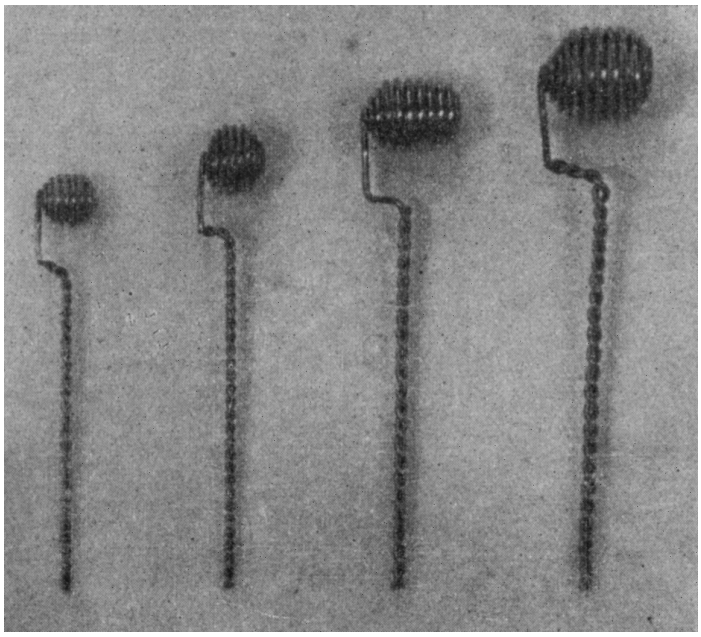

Fig. 1. Spiral loops, holding 0.025, 0.050, 0.1 and 0.2 ml

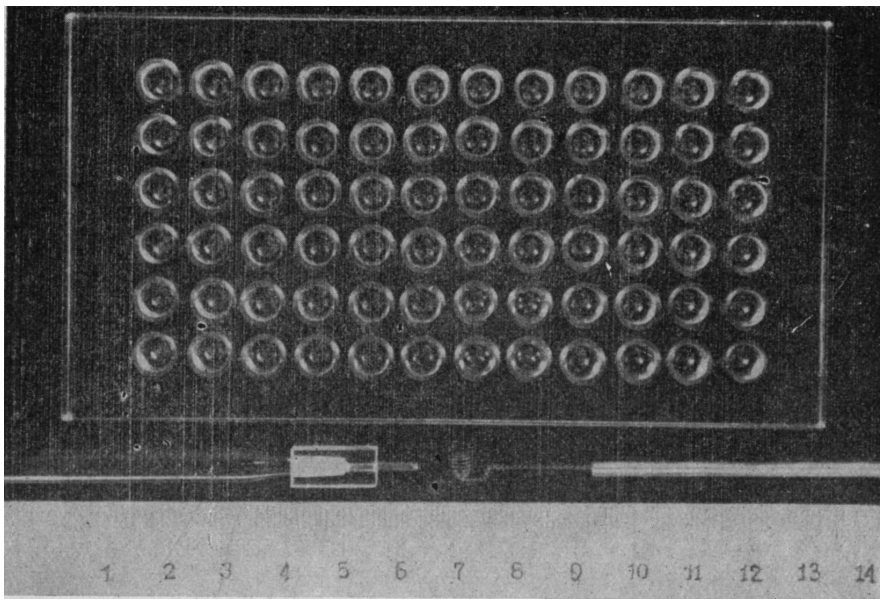

Fig. 2. Above: Plastic plate. On the left, instillator complete with pipette.
On the right, 0.025 ml spiral loop

to be evenly mixed with the diluent. From groove 1 fluid diluted to the required level is transferred to groove 2 and the procedure is repeated until the desired number of dilutions have been made. In practice we usually work simultaneously with 6 loops.

Technique of dilution with the spiral loop

The loops are flamed with a Bunsen burner, partly to sterilize them and partly to remove fat, since a fat-contaminated loop does not take up fluid. The technique of flaming, however, is not identical with that used in bacteriological work. Care should be taken *not* to heat the loop to red heat. The following procedure has proved most suitable for this purpose. Six loops are flamed simultaneously, rotating the holders so as to heat each loop as evenly as possible. If any of the wires begins to glow, flaming is discontinued without delay. After flaming, the loops are allowed to cool. Meanhwile the diluent is measured in the grooves (Fig. 3), or, if there is someone to assist us, it is practicable to work with alternating series of loops. The cool loops are immersed into the diluent (Fig. 4) by which they become filled. The surface of the fluid must not be foamy lest bubbles enter the loop. The loop should not be immersed deep into the fluid; it suffices to touch the fluid with the free tip of the loop, since capillary attraction fills it satisfactorily. Removal

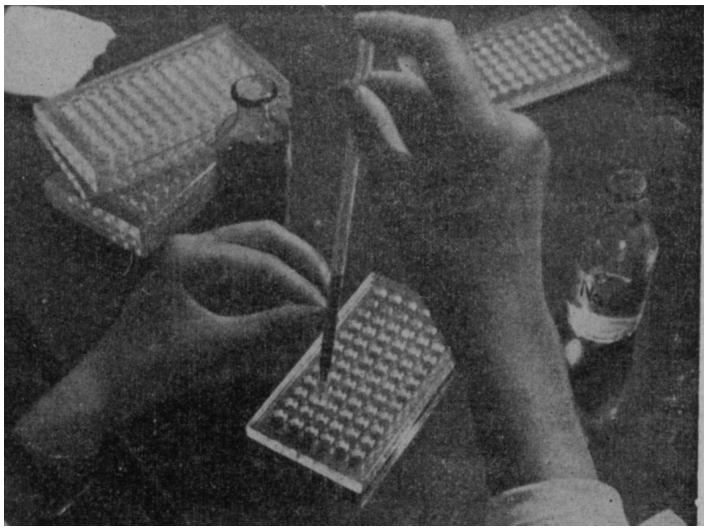

Fig. 3. Dripping in of diluent

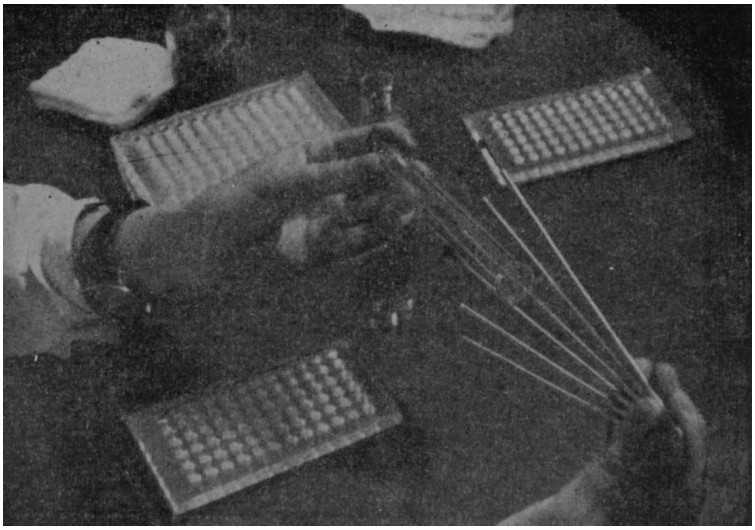

Fig. 4. Uptake of fluid with loop

from the fluid should be carried out slowly or by touching the loop to the wall of the tube. If the loop is withdrawn rapidly a droplet of varying size will remain hanging on it, while an adequately withdrawn loop has no fluid on its outer surface. Sudden shaking or repeated touching of a wet surface by the loop will make it to release the fluid. The loops holding the diluent are introduced into the grooves constituting the first row of the plate, wherein they are rotated firmly for a short time, as described above (Fig. 5). Then the loops are removed from row 1 to make the further dilutions (Fig. 6). Having completed the dilution series, the residual fluid is removed from the loops by means of tissue paper (Fig. 7) and the loops are flamed.

The commonest titration procedures in virus research

Haemagglutination (HA) test. In informative titration, 0.05 ml (two drops) of a 0.5 per cent washed chicken red cell suspension are added to each groove of a plate by means of the instillator attached to an ungraded pipette. Using the 0.025 ml loop, 0.025 ml of the virus suspension to be tested is taken up and diluted serially in as many grooves as desirable. In this case threefold dilutions are made, and the dilution values are 1 : 3, 9, 27, 81, etc. If we want to obtain a precise end point, another series is set up, diluting the first member of this 1 : 5, instead of 1 : 3 (by

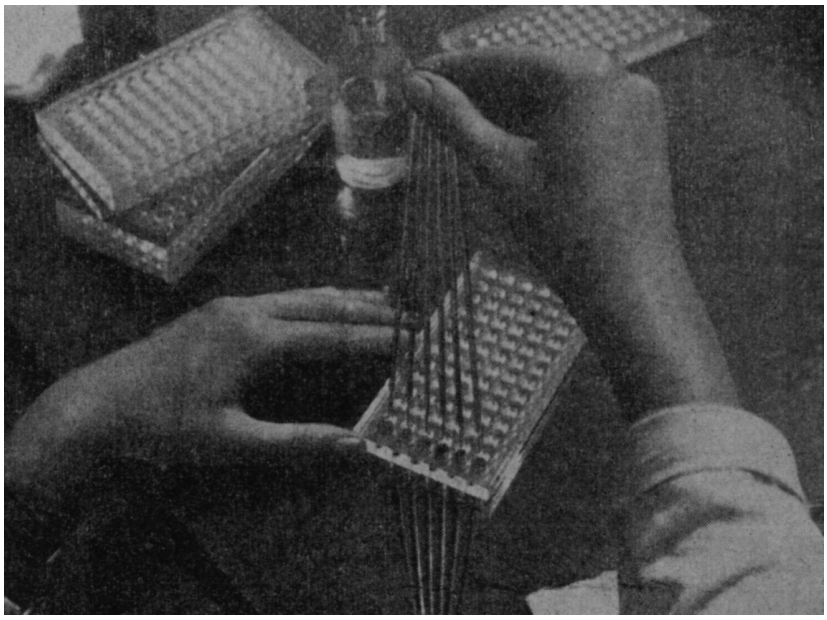

Fig. 5. Insertion of loops into grooves

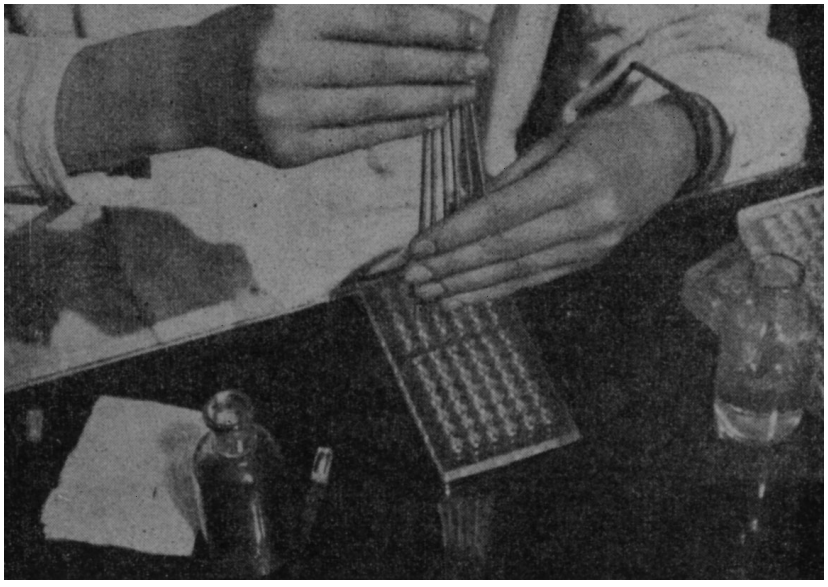

Fig. 6. Transfer of loops in making dilutions

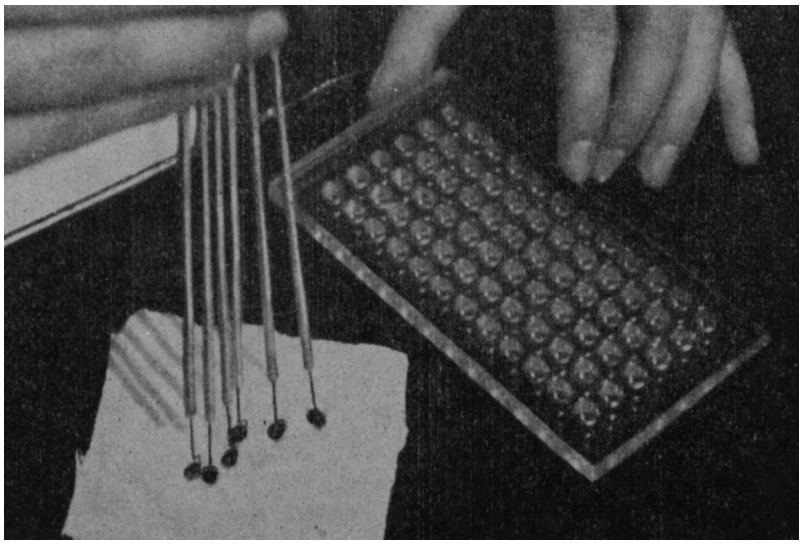

Fig. 7. Blotting of fluid from loops

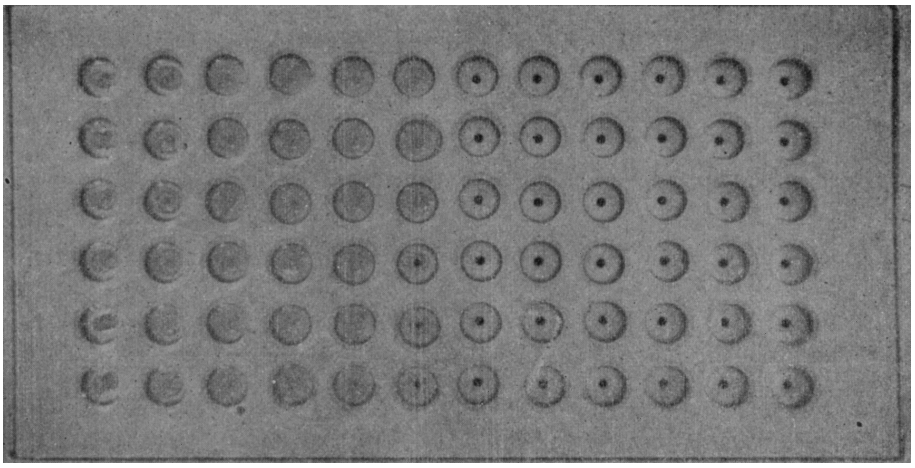

Fig. 8. Appearance of haemagglutination reaction after settling

adding 0.1 ml = four drops to groove one). Thus, the two series will contain dilutions of 1 : 3, 5, 9, 15, 27, etc. The advantage of this parallel titration is that the end point is attained with a smaller number of intermediate dilutions, so that the error will be smaller and still the dilution series will be more protracted than with a two-fold dilution series. After making the dilutions the plates are incubated at room temperature and when using chicken red cells the results are read after 30 to 35

minutes. The appearence of the reaction is shown in Fig. 8. In the presence of agglutination, an even coat of red cell network is seen at the bottom of the groove, as compared with no, or partial, agglutination, where a dense disc of sharp outlines or a smaller disc with agglutination halo is formed. If partial haemagglutination designated with one to three crosses (+, ++, +++) is taken into consideration, an accurate estimate made of the HA titre of the suspension.

A titration of this type is illustrated in Fig. 8, for 3 parallels each. It can be seen that agglutination is complete (+ + + +) at dilution 1 : 729, it is negative at dilution 1 : 2187 (first 3 rows). In the series beginning with dilution 1 : 5 (rows 4 to 6), a one cross (+) agglutination is obtained for dilution 1 : 1215. If the end titre is taken to be the 50 per cent agglutination (+ +), the calculation will be the following. The difference between the reciprocals of the dilutions causing partial (in the above example, +) and total (+ + + +) agglutination is 1215 – 729 = 486. Dividing this by 3 (i e. by the difference in the number of crosses) we obtain the one-cross value (162). Subtracting this from 1215, or adding its double to 729, will give the reciprocal of the dilution causing ++ agglutination, i.e. that of the end point, which in this case is 1053.

Incubation time may be considerably shortened by shaking the plates for one-half to one minute and by centrifugating them in a suitably arranged centrifuge (Fig. 9) at 500 r. p. m. for 10 sec. Then the plates are placed in the vertical position, when agglutinated red cells remain at the bottom of the grooves, while from negative grooves the red cell disc flows out (Fig. 10). Grooves showing partial discharge mark the end point of HA titration.

For determining HA titre, the viral material is diluted directly in the red cell suspension proper. (See above.) This method has been chosen not only because of its simplicity, but also because the results obtained by diluting virus in physiological saline and by subsequent addition of red cells are not easily reproducible, especially when dilute or old viral suspensions are titrated. The error in titrating such suspensions appears to be due to adsorption of virus; the rate of this depends on the quality of the material the loop is made of, or on the condition of the oxidized layer coating it. If virus is diluted in a red cell suspension, the great affinity of the virus to red cells will prevent its adsorption onto the wire. No such adsorption occurs when immune sera are diluted. In the HA test described, in the first members of the dilution series the red cell suspension becomes somewhat diluted by the virus material added. This dilution is negligible in the later members of the series. To avoid dilution of the red cell suspension, to the first grooves of each titration series we may substitute the drop of the 0.5 per cent red cell suspension by one drop of a 1 per cent suspension.

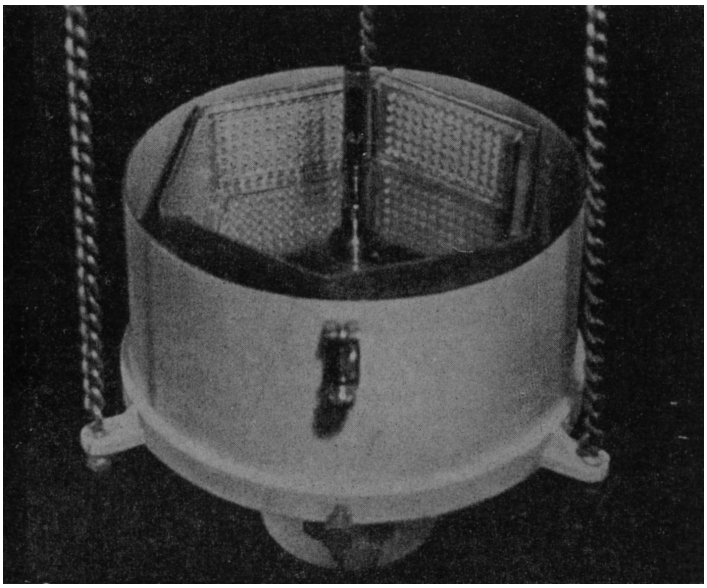

Fig. 9. Centrifuge for plates

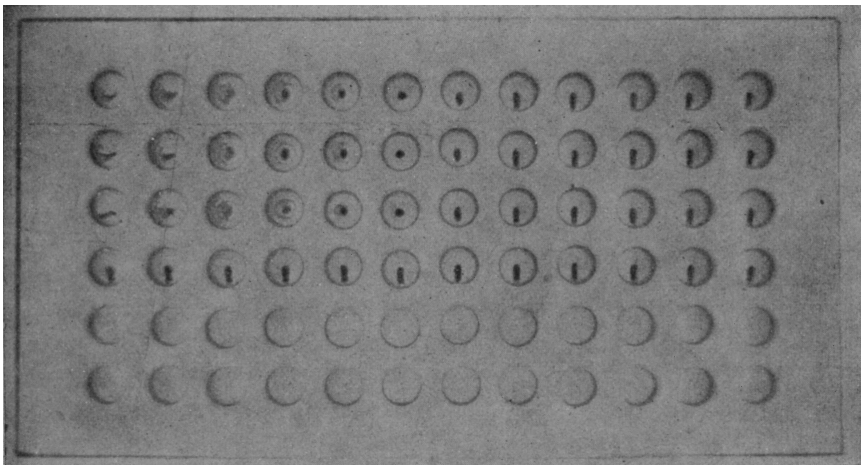

Fig. 10. Appearance of virus haemagglutination after centrifugation

Haemagglutination inhibition (HI) test. Using the 0.025 ml loop, the serum to be tested is diluted in 0.025 ml (one drop) of physiological saline and to each groove are dripped 0.025 ml of a virus suspension containing 8 HA units. Serum and virus are mixed by repeated tapping of the rim of the plates (Fig. 11). After 15

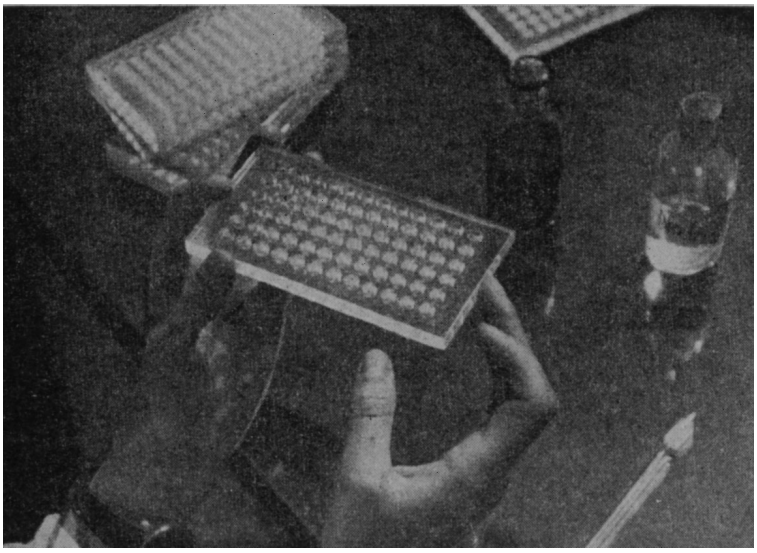

Fig. 11. Mixing of reagents

minutes of incubation at room temperature, 0.025 ml of a 1 per cent red cell suspension are added and the plates are shaken again. After 20 to 30 minutes at room temperature the results are read. A two-cross (+ +) reaction is considered as the end point of HI titration.

The 8 HA units are titrated in the following way. The HA titre of the virus containing allantoic fluid is precisely determined in 3 parallel titrations as described above, except that after completing the dilution series 1 drop of physiological saline is added to each groove. By dividing the reciprocal of the titre by 8, the reciprocal of the dilution desired is obtained. This dilution is titrated again in 3 parallels. The titration procedure consists of the following steps. Into the first grooves of the plate are measured 0.05 ml (2 drops) of a 1 per cent chicken red cell suspension, while to the other grooves a 0.5 per cent chicken red cell suspension is added. Using the 0.05 ml loop, a twofold dilution series is made, then 1 drop of physiological saline is added to each groove. The dilution becomes suitable for inhibition when its titre is 1 : 8, i.e. when the third member of the dilution series gives a two-cross (+ +) haemagglutination. The titre of the viral suspension used for inhibition is checked at the time when the serum is being diluted, or, in prolonged experiments, at frequent intervals.

Complement fixation test. Using the 0.025 ml loop, a serial dilution is made of the test serum in 0.025 ml of physiological saline. After adding 1 drop of antigen

of titrated concentration or a similar amount of complement, the plates are incubated at 37 °C for 20 minutes. After removal from the incubator 1 drop of the haemolytic system (containing equal amounts of a 5 per cent sheep red cell suspension and of titrated haemolysin) is added, and the plates, after shaking, are put back into the incubator for 20 minutes. Lysis, complete or partial, can be clearly visualized by placing the plate before a dark background (Fig. 12). The anticomplementary effect of serum is titrated in the same way, except that instead of antigen one drop of saline is added to each serum dilution. This quantitative titration will provide for an evaluation of specific fixation, despite a lower anticomplementary effect of the serum.

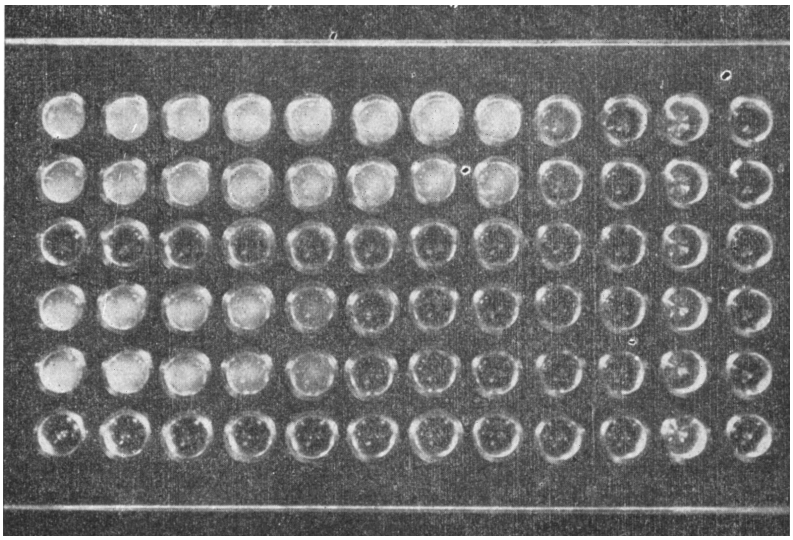

Fig. 12. Appearance of complement fixation reaction

Complement titration is performed in two steps. Of complement diluted 1 : 20, doses increasing by 0.1 ml from 0.1 to 1.0 ml are added to 10 tubes, and the volume made up to 1 ml with physiological saline. To each of 10 grooves in row 1 are added, 1 drop of negative serum diluted 1 : 2 and 1 drop of antigen used in the main reaction. To row 2 are added 1 drop of antigen and 1 drop of physiological saline; to row 3, one drop of negative serum and 1 drop of saline; and to row 4, two drops of physiological saline. At rectangles to each row, 1 drop of each dilution of the complement is placed. After shaking and incubation at 37 °C for 20 minutes, one drop of the haemolytic system is added. After another 20 minutes of incuba-

tion the result is read and the dilution of complement which has caused complete lysis in all four rows is used in the main experiment. If we want the main experiment to be less sensitive, more concentrated complement (by 10 to 20 per cent) should be used.

The cold complement fixation test is carried out by the same technique, except that phase 1 is maintained at +4 °C for 18 hours. The dilution of complement to be used in the reaction is determined by cold fixation.

The complement is preserved by an equal volume of a mixture of 12 per cent sodium acetate and 4 per cent boric acid and is stored in a refrigerator. It will maintain its activity for about 4 to 5 weeks, without any significant decrease in titre.

Sheep red cells are preserved with a modified ALSEVER solution [6].

The advantages of the loop method

First of all, it is much *more rapid* than WIDAL's dilution technique. Dilution is a completely mechanical procedure, not involving tiresome pipetting. Its technique is simple and with little training one can use six loops simultaneously, for diluting different materials or for making several parallels of one material. In general, the reactions can be read sooner and are easy to supervise. The plates occupy less space, can be cleaned rapidly and thus the method is especially advantageous in routine work. (One assistant can carry out 100 to 150 titrations within 1 hour.) Moreover, the method is exact, for several reasons. (1) The volume of fluid measured varies within less wide limits of error than with measuring by pipettes; (2) error due to smearing of more concentrated solutions on the wall of pipette is absent, since the entire surface of the loop that had been in contact with the more concentrated solution is washed into the diluent. Finally, (3) diluting with the loop is perfectly devoid of subjective error of measurement. The method is economical, insofar as it does not involve the use of pipettes and an almost unlimited number of dilution series can be prepared with 12 loops, without being necessary to leave one's seat. There is no washing, preparation or sterilization of pipettes. The required volume of reagents is approximately one-tenth the usual.

The loop is superior to the pipette in every case when minute volumes of fluid have to be measured or diluted. Thus for example, very small volumes of blood taken from the finger tip or from laboratory animals can be taken up and diluted for quantitative work. Several samples of serum can be taken from centrifuged blood without causing thereby the serum to become turbid. The use of cali-

brated loops is advantageous in paper chromatography and electrophoresis, since minute volumes of fluid can be spread out precisely and evenly over the surface of the paper (SZALAY [7]).

In view of the above advantages, the loop technique is now in extensive use, especially for HA and complement fixation tests. The micro complement fixation test has been employed, for instance, by FARKAS and associates [8, 9, 10], by SZÖLLŐSY, IVÁNOVICS and HORVÁTH [11], by SZÖLLŐSY, ÁBRAHÁM and ALFÖLDI [12], as well as by FAZEKAS DE ST. GROTH [13] in influenza virus studies; by DÖMÖK et al. [14] in typhus, by TAKÁTSY and SZAFIR [15] in mumps, by DÖMÖK [16] in Coxsackie virus, by KÖRNYEI [17] in foot-and-mouth disease, and by ELEK and VIZY [19] in brucellosis studies. HORVÁTH and ALFÖLDI [18] have employed the spiral loop technique in their phage titration method, while LÁSZLÓ and SZABÓ [20] have used it in their rapid method for determining antibiotics in a single drop of fermentation fluid. The method has proved of particular value in our immune serum absorption experiments [21, 22], since only minimum amounts were required of costly virus preparations.

Summary

A new serological micro-method has been described, in which serial dilutions are made in grooved plastic plates by means of specially devised calibrated spiral loops, instead of with pipettes. There are loops capable of holding 0.025 to 0.2 ml of fluid. A calibrated instillator serves for measuring in diluent and reagents. A detailed description has been given of the commonest micro-methods used in virus research (HA, Hl, complement fixation tests), as modified by the author.

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Новый серологичеслий микрометод
Дь. Такачи

Резюме

Описываем новый серологический микрометод, по которому серийные разведения производим в углублениях пластмассовой пластинки, применяя вместо пипетки специальную, калибрированную, спиральную петлю. Петлей можно взять 0,025–0,2 мл жидкости. Замер разбавителя и реагентов производится с помощью калибрированного капельника. Таким способом разведения имеется возможность постановки наиболее часто применяемых в вирусологии реакций (реакции гемагглютинации, торможения гемагглютинации и связывания комплемента) с необходимой точностью и при этом достигается также большая экономия материалов и времени.

Editorial notes

Several millions of laboratory workers world-wide use micro-techniques day by day belonging to different fields of natural science. Very few of them know, however, that a Hungarian virologist, Gyula Takátsy M.D., invented the basis of those techniques. He worked at the Department of Virology, National Institute of Public Health established in 1927. After the World War II the laboratory facilities were very poor in that Institute. There was a shortage even in elementary laboratory tools like pipettes and test tubes. That disappointing situation gave an incentive to Dr Takátsy to create simple tools, which may substitute for the classical laboratory appliances. Therefore he invented the microtitration technique with drop-

pers and spiral loops (dilutors) replacing the pipettes as well as plastic plates with wells replacing the test tubes. The technique described in the above paper became widely used all over the world for at least 25 years applying the commercial products of Hungarian and American firms. In the last decades the dilutors and droppers has been replaced by micropipettes or even by automatic dispensers, but the plastic plates with wells (especially that with 96 wells existing since 1956) has remained in use. We think that Gy. Takátsy well deserved to pay tribute to him especially because based on his fundamental invention and initiation the micro-techniques have resulted in enormous development both in research and practice on fields of many branches of natural science.