J. Hyg., Camb. (1962), **60**, 113
With 2 plates
Printed in Great Britain

The measurement of the D-antigen in poliovirus preparations

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(*Received* 20 *July* 1961)

INTRODUCTION

Two antigens have been detected in many poliovirus preparations by complement fixation (Roizman, Höpken & Mayer, 1958) or precipitin tests in agar (Le Bouvier, 1959). Examination of purified concentrated preparations of poliovirus centrifuged in a sucrose density gradient reveals at least two virus-containing zones (Schwerdt & Schaffer, 1956; Le Bouvier, Schwerdt & Schaffer, 1957). One of these, the D-zone, shows most of the infectivity and also reveals intact virus in electron photomicrographs. The virus in this zone contains 25–30 % ribonucleic acid. The antigen from this zone, reacting in precipitin or complement-fixation tests with antibody, is also called D. Antigen in the C zone also reacts with antibody to give a precipitin line and fixes complement. Virus in this zone is of low infectivity and contains little ribonucleic acid. The C- and D-antigens separated by centrifugation in a sucrose density gradient are indistinguishable from those of infected tissue cultures, and the C-antigen is indistinguishable from that of heated poliovirus preparations. Since the C- and D-fractions have morphological and physical characters not unequivocally proved identical with those of heated and native poliovirus preparations, some authors have preferred to call these antigens H and N (Hummeler & Hamparian, 1958). D-antigen (such preparations almost always contain small amounts of C-antigen) gives rise, on inoculation into animals, to formation of anti-D, anti-C and neutralizing antibodies. On the other hand, C-antigen only gives rise to anti-C antibodies detectable in a complement fixation or precipitin test. *In vitro* methods of assaying poliovirus antigens have been hampered by difficulties in distinguishing between these two antigens (Warren, Hare & Goldman, 1959). Infectivity titrations do not measure the inactivated D-antigen present, for example, in ultra-violet-irradiated or formalin-treated preparations. This problem has been solved by means of the complement fixation and use of pure anti-D or anti-C sera (Roizman *et al.* 1958) or by absorption separation of the anti-D component from anti-D and C sera (Hummeler & Hamparian, 1958). Le Bouvier (1959) has shown that these two antigens can be measured simultaneously by precipitin tests in agar gel.

This paper describes a micro-method, based on a technique devised by Crowle (1958), for quantitative determination of D-antigen in the presence of C-antigen by use of precipitin tests in agar.

MATERIALS AND METHODS

Virus preparations

Poliovirus Type 1, Brunenders strain, was grown on monkey-kidney cells by the Connaught Medical Research Laboratories' technique (Farrell, Wood, MacMorine, Shimada & Graham, 1955) or on trypsinized cells. A few experiments were done with the M.E.F.1 strain of Type 2 and the Saukett strain of Type 3 poliovirus. Infectious virus or virus inactivated by formalin as for the preparation of poliovaccine was used.

Precipitin tests for D- and C-antigens

Apparatus required for test

(*a*) Glass microscope slides: 3 in. × 1 in. or 6 in. × 1 in.

(*b*) 1 % 'Oxoid' Ionagar No. 2 or 1 % 'Difco' Bacto agar in phosphate-saline buffer pH 7·2.

(*c*) Phosphate-saline buffer pH 7·2: NaCl: 4·0 gm.; Na_2HPO_4: 0·7 gm.; N/1 HCl: 0·6 ml. Distilled water to 500 ml.

(*d*) Plastic insulating tape (Scotchboy No. 33, 3M Co.) used three layers thick.

(*e*) Perspex templates: 1 in. squares of $\frac{1}{16}$ in. thick Perspex with a central hole and six peripheral holes. The holes are drilled right through with a $\frac{1}{16}$ in. drill and subsequently three-quarters the way through with a $\frac{9}{64}$ in. drill, thus acquiring a funnel shape. Centres of the peripheral holes are 4 mm. from that of the centre hole. Each hole can hold about 0·015 ml.

A few templates made from $\frac{1}{8}$ in. thick Perspex with four peripheral holes round a central hole have also been used. They have proved easier to make than the others, and the holes can be located more accurately.

Templates of both types can be used many times.

(*f*) Pasteur pipettes finely drawn.

(*g*) Holophot photomicrographic apparatus for enlarging the final results.

(*h*) 0·1 % thiazine red in 1 % acetic acid.

(*i*) Air-tight box kept humidified by a wad of wet cotton wool.

Procedure

Two narrow strips of plastic tape are placed across the slides, either 4·5 or 12·5 cm. apart, depending on the size of the slide used. Between the strips is placed molten agar, 0·7 or 1·7 ml., cooled to about 60° C., and a second slide is placed immediately across the top resting on the strips of tape. When the agar is firmly set, the top slide is gently slid off, and the strips of tape are removed, leaving a perfectly flat sheet of agar about 0·6 mm. thick. The Perspex templates are then carefully placed in position on the agar sheet.

Samples to be tested are placed in the holes by means of a finely drawn Pasteur pipette. The holes must be carefully filled from the bottom to avoid air-bubbles. Usually, antiserum is used in the central hole and portions of one antigen preparation in all the peripheral holes. Five templates are required for a quantitative test, or two if the four-hole design is used. Pl. 1 shows two prepared slides.

The prepared slides are placed in a humidified box at room temperature, and the results are read after a minimum of 40 hr., when the templates are flipped off and the slides stained with 0·1 % thiazine red in 1 % acetic acid for 10 min. While the slides are still wet, the images are thrown on to a ground-glass screen by a Holophot photomicrographic apparatus under oblique illumination using a magnification of 8·5-fold. The distance between the leading edge of each precipitin line and the centre of the antiserum hole is measured in millimetres, and an average figure is obtained for each template. The final result is taken as the mean of five (or two four-hole) templates.

Method of concentration of antigens

The routine method involved a two-stage concentration—first with polyethylene glycol (Carbowax 4000 M, Union Carbide Co.) by a method based on that described by Kohn (1959) and then ultracentrifugation in a Spinco 30 rotor. Gelatin (0·06 %) has been added to the virus suspensions before ultracentrifugation to ensure the formation of a pellet (Baron, 1957). The pellet is taken up in the appropriate volume of phosphate-saline buffer, pH 7·2. With a single type of poliovirus a 100-fold concentration is usually employed and for mixtures of all three types a 300-fold concentration.

Antisera

Donkeys were hyperimmunized by repeated inoculations of poliovirus grown on monkey-kidney cells. No monkey-kidney antibody was detected by the gel diffusion test. The optimum dilution for each serum was determined in chessboard titrations with highly concentrated antigens. For routine tests the dilutions in phosphate-saline buffer pH 7·2 used were: Type 1, 1 in 25, Type 2, 1 in 20, Type 3, 1 in 64, and these titred 1 in 16,000, 1 in 32,000 and 1 in 32,000, respectively, in neutralization tests. These sera were heat-inactivated at 56° C. for 30 min. before use.

RESULTS

The appearances of the precipitin lines thrown on a ground-glass screen are illustrated in Pl. 2, figs. 2 and 3. Pl. 2, fig. 2 shows the same D-antigen preparation (the standard control Type 1 material used in the assay of D-antigen) in the six peripheral holes and the standard anti-D and C serum in the central well. A uniform precipitin line is obtained, and it is easy to measure the distance of the leading edge of the precipitate from the centre of the template. Pl. 2, fig. 3 shows the result obtained when a D-antigen preparation is used alternately with C- (D-antigen heated to 60° C. for half an hour) antigen round the centre well containing the same anti-D and C serum. This control antigen preparation, therefore, does not contain C-antigen detectable with the anti-D and C serum. The presence of C-antigen can be detected, however, by using a more potent anti-C serum. With this serum no D-line is obtained, and a line fusing with the C-antigen control line is revealed in the standard D-antigen (Pl. 2, fig. 4). Table 1 and Text-fig. 1 show the results of an experiment in which a living Type 1 virus preparation was diluted serially and D-antigen precipitin tests were performed on the samples. In the figure the distance in millimetres of the leading edge of the precipitate from the centre well is plotted against infectivity. The infectivity was determined on the undiluted preparation, and other results are calculated from knowledge of the dilution. It will be seen that the precipitate moves 2·07 mm. nearer the antigen

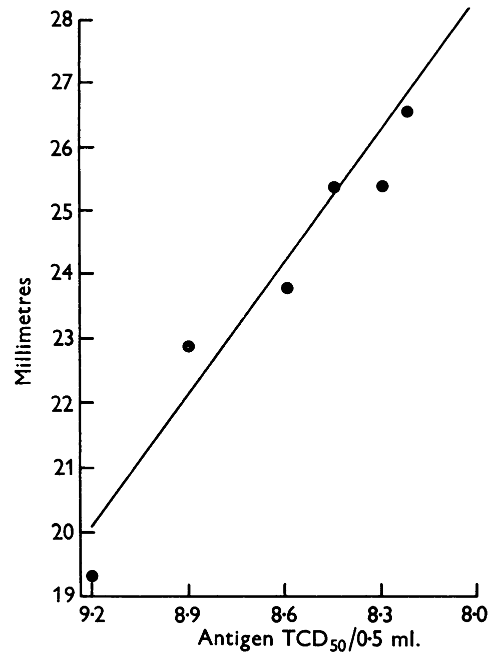
Table 1. *Type 1 poliovirus D-antigen determinations*

Antigen dilution	Individual template readings (mm.) 1	2	3	4	5	6	Template mean (mm.)	Group mean (mm.)
1/1	17	17	18	19	20	19	18·3	19·3
	17	18	18	20	19	19	18·5	
	18	17	18	19	20	20	18·7	
	20	20	20	23	21	22	21·0	
	18	17	20	23	21	21	20·0	
1/2	23	22	25	25	24	24	23·8	22·9
	24	23	23	24	25	25	24·0	
	21	21	20	22	23	22	21·5	
	22	21	21	26	22	23	22·5	
1/4	24	23	23	23	24	24	23·5	23·8
	21	20	24	25	24	24	23·0	
	21	22	24	24	26	23	23·3	
	26	23	25	25	23	28	25·0	
	24	23	25	26	23	25	24·3	
1/6	26	23	24	25	26	24	24·7	25·4
	28	24	23	25	27	26	25·5	
	25	26	27	28	—	26	26·4	
	23	23	27	29	27	26	25·8	
	24	22	25	26	24	26	24·5	
1/8	23	22	26	25	28	28	25·3	25·4
	23	22	25	27	25	26	24·7	
	23	26	26	25	26	24	25·0	
	26	24	30	28	30	27	27·5	
	26	28	24	22	23	25	24·7	
1/10	25	25	26	27	27	26	26·0	26·6
	27	25	26	30	28	29	27·5	
	24	25	26	28	24	25	25·3	
	26	25	29	27	28	27	27·0	
	25	25	30	28	30	25	27·1	

cup for every twofold dilution of antigen. Thus, a preparation giving a D-antigen reading of 22 mm. is approximately twofold stronger than one giving a D-antigen reading of 24 mm. Table 2 and Text-fig. 2 show results for a Type 3 poliovirus preparation. Essentially similar curves can be drawn with Type 2 preparations and for the C-antigen component of all three types (Le Bouvier, 1959), but less work has been done with these.

A straight line, such as that shown in Text-fig. 1 or 2, can be used to assess the infectivity of poliovirus preparations prepared. Some examples are shown in Table 3. It will be seen that, in general, there is good agreement between the estimate of infectivity from the D-antigen assay and from direct titration.

D-antigen exists in two forms, infectious (ID) and non-infectious (NID) (since ultra-violet- or formalin-treated preparations of virus still contain D-antigen, even when the infectivity is completely inactivated), so that the validity of these assays depends upon a constant ID/NID ratio. This holds for a standard method of virus production but not if the procedure is varied; thus, for example, if virus is harvested late, at 37° C., more inactivation of infectivity occurs than of D-antigen.

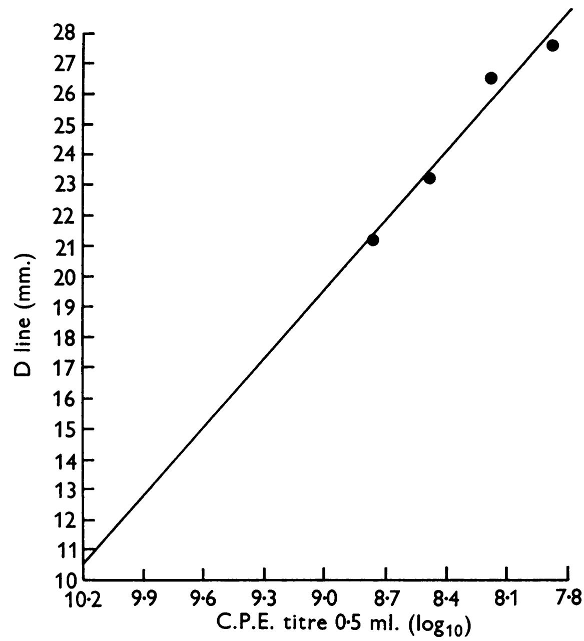


Text-fig. 1. Infectivity of Type 1 poliovirus preparation plotted against the distance of precipitin line from antiserum hole.

Table 2. *Type 3 poliovirus D-antigen determinations*

Antigen dilution	Template mean (mm.)					Group mean (mm.)
1/1	20·8	21·2	21·5	22·0	20·7	21·2
1/2	23·2	23·4	22·2	23·2	23·8	23·2
1/4	27·3	26·8	26·0	26·7	26·5	26·7
1/8	27·7	27·2	27·7	—	—	27·5

In routine assays it is desirable to express the potency of samples under test in terms of a standard. It is necessary to demonstrate that the dose-response relationships for different samples are linear and parallel and that the slope is large enough in relation to random errors of individual responses to provide precise estimates of potency. In a routine assay many slides are required and slide-to-slide variation had therefore to be investigated. An experiment was performed to assess the potency of five preparations, each being examined at three dilutions. The scheme of the experiment is shown in Table 4. The samples were labelled A–E, the three dilutions 1, 2, and 3 being respectively 1, $\frac{3}{4}$, and $\frac{9}{16}$. Each template contained one sample at one dose level. Each of the 30 slides contained all five samples: 10 slides had two samples at dose level 1, two at dose 2 and one at dose 3. Similarly, another 10 had two samples at doses 2 and 3 and one at 1, and finally, another 10 had two at doses 1 and 3 and one at dose 2. Each dose level appeared ten times. Any two samples at one dose level were compared on two slides. Also any one sample at one dose level was compared with any other sample at any other dose level on four slides.



Text-fig. 2. Infectivity of Type 3 poliovirus preparation plotted against the distance of the precipitin line from antiserum hole.

Table 3. *Correlation of infectivity determined by titration in tissue culture and by D-line assay for Type 1 poliovirus*

	Method of titration ($\log_{10}$ TCD_{50} per 0·5 ml.)		
Preparation	D-line	C.P.E.	Difference
1	8·7	8·0	+0·7
2	8·2	7·9	+0·3
3	9·4	9·4	0
4	6·5	6·5	0
5	8·3	8·1	+0·2
6	8·9	8·1	+0·8

This experiment, the results of which are shown in full in Table 5, showed that there were significant differences between slides and that these, although not great, show the need for using a design allowing for inter-slide variations. The samples produced straight parallel dose-response lines. The mean slope was found

Table 4. *Design of experiment to assess value of method for routine assay*

Slide 1	2	3	4	5	6	7	8	9	10
C1	C2	E1	D3	B1	E3	D1	B1	E1	C3
E1	E1	D3	B2	E3	C2	A3	D2	A2	D1
B3	A3	C3	C1	C3	A3	B2	A1	D3	A3
D2	B2	A2	E2	A1	B1	E1	C3	C3	B2
A3	D1	B1	A1	D2	D2	C3	E2	B2	E2

Slide 11	12	13	14	15	16	17	18	19	20
A1	D2	D1	D3	B1	A3	D1	E1	C2	C1
D3	C1	C3	B3	A3	B3	B3	D2	A2	E2
B2	B2	E2	A1	D3	C1	E2	A1	E3	D3
C2	E1	B3	C2	C1	D2	C3	C2	D1	A2
E3	A3	A1	E1	E2	E2	A2	B3	B1	B1

Slide 21	22	23	24	25	26	27	28	29	30
D2	B2	E3	A1	E1	C2	C2	C2	A2	A2
A2	A1	B2	E3	A2	D1	E2	B1	C3	E3
B3	E3	A2	B2	D3	A1	B1	D1	E1	D1
C1	D2	C1	D3	C2	B3	D3	A3	B1	B3
E3	C3	D1	C1	B3	E2	A3	E3	D2	C1

Table 5. *Results of experiment to assess value of method for routine assay. Mean distance in mm. moved by D-antigen in individual templates*

	A1	A2	A3	B1	B2	B3	C1	C2	C3
	24·7	26·2	27·5	22·7	22·5	24·5	20·0	18·1	21·0
	25·5	25·2	26·2	22·2	24·2	23·8	20·8	20·3	21·2
	25·5	25·7	25·7	23·0	22·4	23·8	19·8	19·7	21·2
	25·4	26·7	27·2	22·2	22·7	25·5	21·4	19·8	19·8
	24·0	24·5	26·2	22·7	22·7	23·0	19·3	18·8	21·0
	25·0	24·5	26·2	21·2	22·7	24·0	20·1	20·1	20·1
	25·4	24·0	25·5	23·0	23·4	25·5	19·8	19·8	20·7
	24·2	27·0	25·8	21·8	21·8	26·8	20·5	21·0	21·2
	25·2	27·0	26·0	22·5	23·0	25·7	20·0	19·5	20·5
	25·4	—	27·3	23·6	23·5	24·2	19·8	21·0	21·4
Average	25·0	25·6	26·7	22·5	22·9	24·7	20·2	19·8	20·9

	D1	D2	D3	E1	E2	E3
	23·5	25·4	26·7	21·4	21·4	22·0
	23·0	25·0	25·4	19·8	21·0	20·0
	22·8	25·2	26·0	19·8	20·5	21·8
	23·8	24·4	25·7	19·5	19·3	21·7
	23·6	24·4	26·4	20·8	21·8	19·5
	22·4	26·5	26·5	17·8	20·8	20·3
	23·4	23·8	25·4	19·7	21·0	22·7
	25·0	23·6	25·8	19·5	21·7	21·8
	24·5	25·5	25·5	20·1	22·8	21·2
	—	24·5	26·0	20·0	—	20·1
Average	23·6	24·8	25·9	19·8	21·1	21·1

to be 1·95 mm. for a twofold dilution, with 95 % confidence limits of 1·825 mm. to 2·07 mm.

All the samples tested were living virus preparations, and their infectivity was determined by infectivity titrations. The potencies of the preparations in terms of sample A are shown in Table 6. It will be seen that there is good agreement between the two methods of determination. This experiment demonstrates that the precipitin test for D-antigen content as performed is valid. In a routine method with two doses each on five templates the limits would be about $\pm 0{\cdot}12$ log units, given a reasonable preliminary estimate of potency.

Table 6. *Potency of samples in terms of a control by D-antigen and infectivity measurement*

	D-antigen		Infectivity	
Sample	Potency units	95 % confidence limits	Potency units	95 % confidence limits
A	1	—	1	—
B	2·3	1·9–3·0	1·1	0·2–1·7
C	7·0	4·9–11·7	6·2	4·1–9·4
D	1·4	1·2–1·7	0·15	0·02–1·2
E	6·1	4·4–9·7	6·2	4·1–9·4

DISCUSSION AND SUMMARY

A simple micro-method of measuring 'D-' and 'C-' antigen contents of poliovirus preparations is described. The D-antigen of poliovirus is almost certainly the component of the virus involved in eliciting neutralizing antibodies, and the D-antigen assay thus becomes a simple *in vitro* method for assessing the antigenic potency of poliovaccines and for investigating the influence of various factors upon the antigen. The value of the technique in following the concentration and purification of poliovirus preparations is illustrated in the studies reported in the accompanying paper by Fantes (1962). The results of experiments designed to test the validity of the idea that D-antigen is the antigen eliciting neutralizing antibodies are discussed by Beale (1961).

The design of the experiment shown in Table 4 was devised by Mr J. P. R. Tootill, who, with Miss E. M. J. McMullin, also carried out the statistical analysis. The accurate and painstaking technical assistance of Mr J. Green is gratefully acknowledged. Mr Green was also responsible for the photographs of the precipitin lines.

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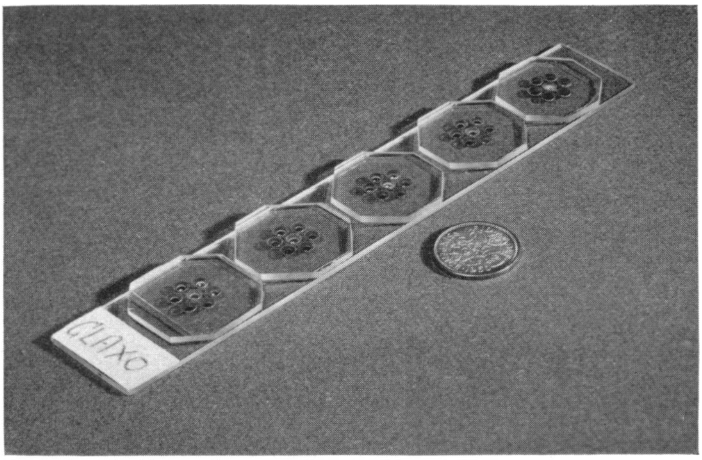

Fig. 1*a*

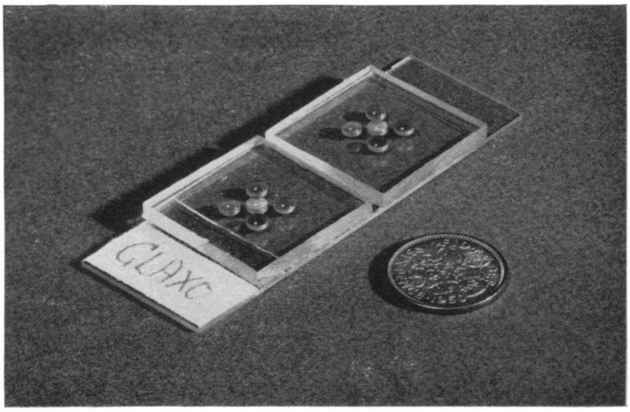

Fig. 1*b*

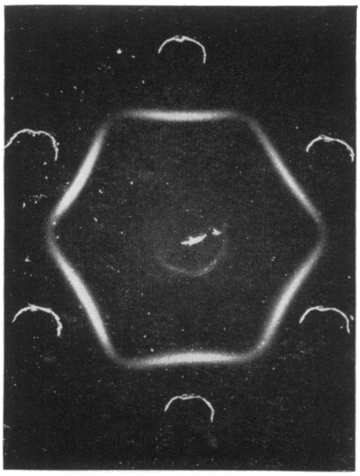

Fig. 2

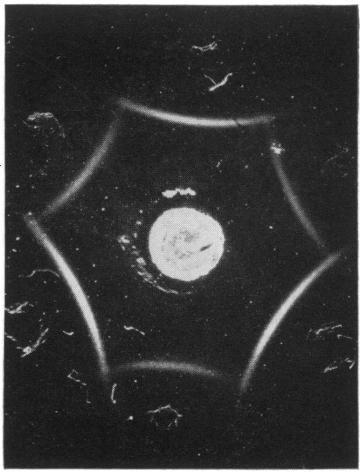

Fig. 3

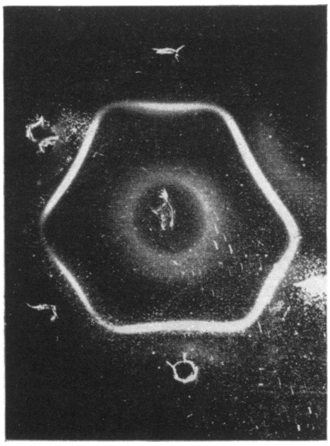

Fig. 4

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Templates set up on agar on a slide.

PLATE 2

Fig. 2. Poliovirus antigen D-precipitin lines. Poliovirus (control D-antigen) preparation in peripheral holes, anti-D and C-serum in the centre.

Fig. 3. Alternate D- and C-precipitin lines. D-antigen as in Fig. 2. C-antigen living poliovirus (control D-antigen) heated to 60° C. for 30 min..

Fig. 4. C-precipitin lines. Same virus preparations as in Fig. 3 with anti-C serum in central hole.