POLIOVIRUS PRECIPITINS

A Study by Means of Diffusion in Agar*

By GEORGE L. LE BOUVIER‡, M.D.

(From the Section of Epidemiology and Preventive Medicine, Yale University School of Medicine, New Haven)

Plates 90 and 91

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The group of recognized human poliomyelitis viruses (*Poliovirus hominis*) is divided into three serological types, distinguished on the basis of their neutralization by hyperimmune antisera. There is increasing evidence, however, for the existence, both of varying degrees of antigenic relationship among strains of different type, and of antigenic differences between strains of the same virus type (1–9). This diversity of antigens may be accompanied by differences in particle size and response to treatment with acetone, formaldehyde, heat, or ultraviolet light (5, 7, 8, 10–14). Virus antigens modified by exposure to such agents can fix complement with antibodies that are not detected by the unmodified antigens; and the converse is also true (7, 8, 13, 14). Distinct antigenic components, moreover, have been separated by various methods of fractionation in the process of poliovirus purification (12–15). The antibodies reacting with these different kinds of antigens tend to differ in their behavior in time as well as in their reactivity. Their definition, and that of their specific antigens, have an important bearing on the serological diagnosis of human poliovirus infection.

The method of antigen-antibody precipitation in gels seemed promising, both as an instrument for antigenic analysis, and as a potential test for a previously unexamined class of poliovirus antibodies. It had already proved useful in studies of the viruses of influenza (16), the pox group (17), and foot-and-mouth disease (18). While it was under investigation, Wilson Smith and his colleagues reported the applicability to poliovirus systems of tube flocculation tests using liquid reactants (19, 20). This paper describes the formation of poliovirus antigen-antibody precipitates in agar, and presents some results concerned with the testing of human sera by this method.

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Materials and Methods

Viruses.—Liquids were obtained from cultures of *rhesus* monkey kidney showing advanced degeneration after infection with one or another of the following strains of poliovirus: Brunhilde, Mahoney, Mexico, and W-S (Type 1); Y-SK and MEF-1 (Type 2); Leon and Saukett (Type 3). The concentrations of infective virus in three large collections of Mahoney, Y-SK, and Saukett culture liquids were $10^{7.3}$, $10^{6.8}$, and $10^{7.5}$ plaque-forming units per ml., respectively.[1] In the presence of optimal concentrations of homotypic monkey antiserum, the endpoint dilution of the Mahoney and Saukett liquids, as unheated complement-fixing antigen preparations, was in both cases 1/4.[2] If a lot of liquid was no good as a preparation of complement-fixing antigens, it was never worth spinning for use in precipitation tests. As a control, liquid from uninoculated monkey kidney cultures was first used. This was later replaced by liquid from cultures infected with the Grigg strain of Type A9 Coxsackie virus, on the ground that this would provide a better control for material obtained from degenerated cultures. The Grigg strain was selected because it has a cytopathogenic effect on monkey kidney cells similar, in several respects, to that of poliovirus (23).

The cultures were made either from minced, suspended tissue grown in Mixture 199 (24) without serum, or from trypsin-dispersed cells grown in 0.5 per cent lactalbumin hydrolysate in Hanks's balanced salt solution, containing 2 per cent calf serum (25). At first, cultures grown in serum were washed twice with Hanks's solution and given the same medium without serum before inoculation with virus. Later, the washing was omitted: in general, it appeared to be unnecessary; and it proved to be ineffective, in any case, in removing all traces of serum.

Control and test liquids were clarified by spinning at 1500 R.P.M. for 15 minutes, and then concentrated by spinning in the No. 40 rotor of a Spinco model L preparative ultracentrifuge for 3 hours at 40,000 R.P.M. (average R.C.F., 100,000 *g*). The deposit was thoroughly resuspended in 1/50 of the original volume of medium, and the resulting opalescent or slightly turbid liquid constituted the antigen-containing reactant, which was stored in small aliquots at −50°C., in an electric refrigerator. Such 50-fold concentrates were not respun after thawing, and were used unheated and undiluted, except as indicated.

Sera.—Tests were made with human and monkey sera. They were used undiluted, unless otherwise stated. A few sera, both human and monkey, were heated at 60°C. for 20 minutes, without any detectable reduction in their content of poliovirus precipitins. In general, they were used unheated.

Monkey Antisera.[3]—The initial experiments were done with several different monotypic pools of serum from *rhesus* monkeys (*Macaca mulatta*) hyperimmunized with poliovirus grown in *rhesus* kidney cultures. Later, one pool was chosen as a "working standard" antiserum for each virus type, after comparison with samples of the antisera produced and described by Wenner *et al.* (26), which were taken as the final standards of reference. The homotypic neutralizing antibody titers of the "working standard" antisera, as determined in panel color tests (25), and the virus strains used as immunizing inocula, were as follows: Type 1 (Brunhilde), 2000; Type 2 (Y-SK), 500; Type 3 (Leon), 8000. None of these monkey sera had any demonstrable heterotypic neutralizing antibody, with the exception of the Type 1 pool, which had a titer of 35 against Type 2 virus. Control sera comprised a pool of normal

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[1] The results of the Mahoney and Saukett titrations were kindly communicated by Dr. Edward M. Opton.

[2] I am indebted for this information to Mrs. Sarah Melnick. The determinations were made by the plate method of Fulton and Dumbell (21), as used in this laboratory (22).

[3] The monkey sera, and information concerning them, were kindly supplied by Dr. Joseph L. Melnick and Miss Jean E. Emmons.

rhesus monkey serum, and serum from monkeys inoculated with one or another of the following viruses grown in cultures of *rhesus* kidney: Coxsackie Type A9 (Grigg strain); ECHO Type 7 (Garnett strain); ECHO Type 9 (Quigley strain). The reference antisera (26) were kept as freeze-dried aliquots, and in small amounts as reconstituted serum, at 4°C. The "working standard" antisera were stored in aliquots at −50°C., and at 4°C. There was no appreciable fall in poliovirus precipitin content during a period of 4 months at 4°C.

Human Sera.—Tests were done on paired serum specimens from 30 individuals, and on single specimens from 7 others. These sera had been stored at −20°C. Of the 30 pairs of sera, 21 were acute- and convalescent-phase specimens from poliomyelitis patients (12 with Type 1, 9 with Type 3 infections);[4] while 9 were pre- and post-feeding specimens from people taking part in a study on the effect of pre-existing immunity, either natural or induced by formalinized vaccine, on the fate of orally administered attenuated virus (27).[5] Some of the patients' sera had been examined for poliovirus neutralizing antibodies in tube tests (28), the remainder, together with those of the participants in the vaccine study, in panel tests (25). Complement-fixing antibodies had been titrated with unheated or heated virus preparations, or with both, by the method in use in this laboratory (22).

Diluent.—All dilutions, suspensions, and solutions, unless otherwise stated, were made in 0.14 M sodium chloride in demineralized distilled water, containing 0.01 M phosphate buffer (pH 7.1–7.2). In the early tests, unbuffered saline was used; but its pH frequently fell to below 6.5, a range in which precipitate formation by poliovirus reactants begins to diminish.

Gel.—After a number of trials, the gel finally chosen consisted of 0.5 per cent *w/v* Difco "Noble" agar made up in the diluent, and sterilized by autoclaving at 10 pounds' pressure for 10 minutes. No preservative was added. The agar was soft enough to permit adequate diffusion of the poliovirus reactants, but firm enough to prevent fuzziness and mechanical disturbance of the precipitate. Antimicrobial compounds were usually omitted. In the initial experiments, crystalline potassium penicillin G, streptomycin sulfate, and mycostatin were added to the agar before plates were poured, to give final concentrations of 100 units per ml., 100 μg. per ml., and 100 units per ml., respectively; but precipitates were generally clearer and more sharply defined when these substances were absent. In some experiments, merthiolate was added to the agar to reduce contamination; and this led to the observation of its deleterious effect on precipitate formation.

"Ring" Precipitin Tests.—All "standard" reactants were first examined in "ring" tests, by layering virus preparation upon serum in capillary tubes. Positive reactions were often visible within a few minutes. Tests were read after ½ to 1 hour at room temperature.

Agar Diffusion Tests in Tubes.—In the first stages of this study, a positive result with the "ring" test led to examination of the reactants by a method of double diffusion in agar based upon that described by Oudin (29). Glass tubing, of internal diameter 2.5 mm., was cut into lengths of 6 cm. Enough agar was introduced to make a "reaction column" 10 mm. long in the middle of the tube, and similar quantities of the two liquid reactants were placed in contact one with each end of the column. The tube was then sealed with wax and kept at 4°C. Readings were made at 3- to 4-day intervals for 3 weeks.

Agar Diffusion Tests in Plates.—At a later stage, when larger amounts of virus concentrates had been prepared, the one dimensional tube tests were superseded by tests of double diffusion in two dimensions (30, 31). These were done in Petri dishes, and the reactants diffused from cups made in the agar. This method, although somewhat more expensive in re-

[4] These sera, and the information concerning them, were kindly provided by Dr. Mary Godenne McCrea and Dr. David C. Davis.

[5] These sera, and the information concerning them, were kindly provided by Dr. Dorothy M. Horstmann and Dr. James C. Niederman.

actants, permitted a direct qualitative, as well as quantitative, comparison of the precipitates formed by different combinations of reactants, in one and the same agar menstruum.

Many variations were made in the conduct of these tests, during the course of the experiments here reported. In the earlier ones, diffusion of the reactants took place at 4°C., for the reason that precipitates, though slower to form, are often more sharply defined at lower temperatures. In these tests, no attempt was made to maintain a constant degree of humidity in the Petri plates: the resulting slow evaporation permitted recharging of the cups at intervals during the relatively prolonged observation period (3 weeks or more), with a consequent increase in concentration of the reactants, and enhanced density and detectability of the precipitates. The changes in agar and salt concentration, and the unevenness of the reactant concentrations (*cf.* reference 29), were apparently not such as to vitiate the qualitative comparisons aimed at in these earlier experiments. The method finally adopted, however, offered greater speed and convenience, as well as a better possibility of controlling test conditions for purposes of assay. This method will be described in detail.

In the "*standard*" *method* for the two dimensional, double diffusion test, 3 ml. of 2 per cent *w/v* agar is first introduced as a foundation layer into a Petri dish 5 cm. in diameter. This provides an even, horizontal floor for the cups, and obviates the seepage of reactants between agar and glass not uncommonly seen in earlier tests. This substratum, once gelled, is overlaid with 7 ml. of 0.5 per cent agar. Cylinders are cut in the upper layer of agar with cork borers, and cups made by sucking out the soft agar with a Pasteur pipette. If the plates are left standing at a cool room temperature for several hours, or preferably overnight, the agar plug is usually just firm enough to be lifted out *en bloc*. (Moulds have been given a trial; but, in agar of this consistency, the cups that they form have rarely been as neat and uniform as those made by the method described.) The cups are 3.5 mm. deep. Those intended for serum are 3.5 mm. in diameter. Wider cups, 5.5 mm. in diameter, are used for virus concentrates, in order to provide a larger volume of reactant relative to the area of the sides of the cup. In some of the early tests, such as the test shown in Fig. 2, the smaller cups were used for virus. The volume of the serum cups is 0.034 ml.; that of the virus cups, 0.083 ml. They are so arranged that the shortest distance between the rim of any given serum cup and that of each neighboring virus cup is 10 mm. Any diluent collecting in the cups is sucked out just before they are filled to the brim with reactant. When the results obtained by introducing measured volumes into the cups are compared with those obtained by merely filling them to the brim, the former, more laborious procedure appears to have no special advantage over the latter, at least as far as the present kinds of test are concerned. The charged plates are placed in an humidified jar at 35–37°C. Since the cups are not refilled, 0.12 ml. of undiluted serum is enough for the assay of precipitins against the three types of poliovirus; and 0.09 ml. of 50-fold virus concentrate of each type (or 4.5 ml. of original tissue culture liquid) suffices to test paired serum specimens from two patients, together with the reference serum in parallel, as described in the section on Results.

Reading of Tests.—The plates are read at varying intervals, usually after 3, 5, and 7 days' incubation at 35–37°C. The precipitates are viewed by oblique light of variable intensity. For examination, a plate is held horizontally, and the light source, screened from direct view, is placed below and to one side of it, so that the incident beam makes an angle of about 30° with the perpendicular. The character of a precipitate is noted, and the distance of its leading edge from the relevant virus and serum cups is measured with a pair of dividers. The position taken up by the precipitate band, within the 10 mm. interval separating the two cups, provides an indication of the relative concentration of homologous antigen and antibody in these cups; so also do its degree of sharpness and its tendency to migrate; while its size and density reflect the absolute, as well as the relative, concentration of the reactants.

As a rule, the 5-day readings have been taken as the standard values for the reference sera; and definitive readings for unknown sera and other reactants under test have therefore usually been those made on the 5th day of incubation.

RESULTS

Precipitate Formation by Polioviruses and Homotypic Antisera

Within a few minutes after the setting-up of a "ring" test, certain poliovirus concentrates and homotypic monkey antisera can be seen to be forming a milky disc at their interface. Such homotypic reactants, when allowed to diffuse into agar, produce a turbid band wherever they happen to meet in equivalent proportions and in sufficient concentration. In double diffusion tests done in tubes, the point in the 10 mm. long agar "reaction column" at which a precipitate forms is a measure of the relative concentration of the interacting antigen and antibody diffusing into the column from either end (see references 29 and 32). The same applies to plate tests, except that here the 10 mm. column is represented by the 10 mm. wide "reaction arena" that separates the virus and serum cups.

The dependence of the position of the precipitate band on the relative reactant concentrations is readily seen in tube tests.

Fig. 1 shows a titration of the Type 1 (Brunhilde) monkey serum pool against Type 1 (Mahoney) poliovirus. The upper parts of the tubes contain serial twofold dilutions of serum; the lower, the same 50-fold virus concentrate throughout. Two bands have developed in the portion of the agar column nearest to the virus depot: the band further from this depot, the more prominent of the two, has been called the "major" band; the other, the "minor" band. As can be seen in Fig. 1, serial twofold dilution of the monkey serum causes the bands to become progressively more tenuous and closer to the serum depot. (The diagram which accompanies the photograph, in this and in later figures, is designed to clarify the picture: it emphasizes the antigen-antibody precipitates and ignores the areas of opacity produced by either reactant alone.) The photograph in Fig. 1 was taken after the tubes had been 7 days at 4°C. At this time, in the tube containing the undiluted serum, the meniscus of agar in contact with the virus concentrate was clear. During the succeeding 2 weeks, however, the position of the "zones of precipitation" shifted slowly towards the virus depot, so that the lower, minor band came eventually to be represented by an incrustation of precipitate adhering to the surface of the meniscus.

It may be mentioned here that comparable pairs of precipitate bands have been observed with strains of each of the three virus types; and that both bands have been formed only by homotypic combinations of virus and antiserum. Their detection depends on the presence in the serum of enough antibody against each of the antigenic components. Clearly defined minor bands have not been seen with the human sera so far tested. Using a given monkey antiserum, variations in the relative position and prominence of the major and minor bands have been the only differences hitherto noted between dif-

ferent strains of the same virus type; and similar variations may also be observed between different lots of concentrate prepared from a single strain. With some preparations, only a single, major band of precipitate has been formed.

Specificity of the Poliovirus Precipitation Reaction

The type-specific character of the precipitates formed by poliovirus concentrates and homotypic antisera is shown in the plate test illustrated in Fig. 2.

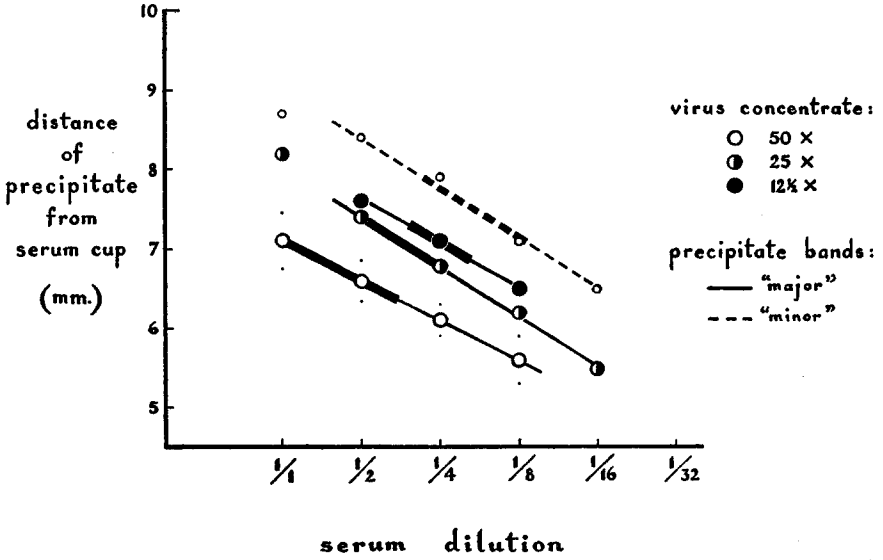
Poliovirus of Types 1, 2, and 3 is represented by 50-fold concentrates of the Brunhilde, Y-SK, and Leon strains, respectively. A concentrate of Type A9 Coxsackie virus (Grigg strain) is included as a control of degenerated monkey kidney. Certain precipitates have been formed only between each poliovirus cup and the homotypic serum cup. The one apparent exception was a very faint precipitate, not discernible in the photograph, produced by the Type 1 antiserum with the Type 2 virus. This was presumably a specific Type 2 precipitate, and not an example of a cross reaction; for this Type 1 antiserum was found to have a low titer of Type 2 neutralizing antibody.

There is nothing in Fig. 2 suggestive of a "group-specific" precipitate, common to two, or to all three, poliovirus types; and none such have been observed in subsequent experiments. But there are pairs of non-specific bands, seen also with the Coxsackie-virus control, which were later attributed to a reaction between antibodies in the Type 3 antiserum against calf serum constituents and traces of calf serum remaining in the virus preparations. In further experiments, the same poliovirus concentrates formed no precipitates, either with serum from uninoculated monkeys, or with antisera against Coxsackie A9, ECHO 7, and ECHO 9 viruses. Varying the dilutions of antisera and of virus concentrates did not result in the appearance of any heterotypic or non-specific precipitates.

Standardization of Reactants for Tests of Human Sera

A type-specific precipitation reaction was therefore available with which to look for poliovirus precipitins in human serum. It was soon evident that these might be present in large amount in patients with high titers of neutralizing antibody of the corresponding type. To assay these precipitins, a monkey serum pool of each type was chosen as a "working standard," as described above. This served to characterize new lots of virus concentrates. It was included in every test of unknown sera, as a means of measuring their precipitin content, and as a check on the virus dose. Fusion of precipitate bands, in a plate test, may usually be taken as evidence of the effective identity of the antigen-antibody systems concerned: so the "standard" serum also controlled the specificity of any precipitates that might be formed by the sera under test.

"Standard" antisera were first examined in chessboard titrations against varying dilutions of a particular lot of homotypic virus concentrate, containing either the Mahoney (Type 1), the Y-SK (Type 2), or the Saukett strain (Type 3). The graphic record of one such chessboard titration of Type 1 reactants is shown in Text-fig. 1: the curves for both the major and the minor bands of precipitate are included. It is the major band, however, that is formed by



TEXT-FIG. 1. Chessboard precipitin titration in agar of "standard" Type 1 (Brunhilde) monkey antiserum against Type 1 (Mahoney) poliovirus concentrate. Reactants unheated. Readings are those made after 5 days' incubation of the test plates at 35–37°C. Distances plotted on the ordinate indicate the position of the type-specific antigen-antibody precipitates within the 10 mm. interval of agar separating the serum and virus cups. Dots above and below points on the major-band curve for the 50× virus concentrate indicate 95 per cent confidence limits of readings. Thickened segments of curves mark the range of serum dilutions giving the sharpest and densest band of precipitate with a particular virus concentration.

human sera with the virus concentrates used in these experiments; and all ensuing observations on human sera relate to this band.

The highest dilution of the "standard" monkey antiserum capable of producing a clear, major band of precipitate at a distance of between 6.0 and 6.5 mm. from the serum cup, with any dilution of the virus preparation, was arbitrarily taken as containing 1 unit of type-specific poliovirus precipitin per unit volume (*i.e.*, per 0.034 ml., the volume of the serum cups). In tests of human sera, two dilutions of the homotypic "standard" serum were tested in parallel on each plate carrying virus concentrate of a given type. These dilutions were selected to contain 1 and 4 units, or sometimes higher values, of precipitin per unit volume. Since reference sera were present on each plate, and since the slopes of the lines for the different virus concentrations were approximately equal (Text-fig. 1), the exact dose of virus

on a given plate was not critical: the undiluted, *i.e.* 50-fold, concentrates were generally used, so as to obtain precipitate bands of maximum density.

The feasibility of measuring poliovirus precipitins in unknown sera by the plate method, as here described, depends on the empirical finding that, with a constant dose of any of the three virus types, there exists a linear relationship between the logarithm of the serum concentration and the distance from the serum cup at which the band of precipitate is formed. This holds for human as well as for monkey sera. (For a theoretical discussion of such a relationship, see reference 32.)

With the Type 1 reactants whose chessboard titration is shown in Text-fig. 1, if the serum concentration was halved, then the major band of precipitate developed at a point about 0.5 mm. closer to the serum cup. A comparable distance was found for other combinations of reactants; but sometimes it was larger, up to 0.75 mm.

The precipitin content, P, of an unknown serum, in units per unit volume (0.034 ml.), is calculated from the formula

$$P = D^{\left(\frac{x - x_s}{a}\right)}$$

in which D is the serial dilution factor used in titrating the "standard" serum (= 2, in all of the present experiments); x_s is the distance in millimeters from the serum cup of the major band formed by the dilution of "standard" serum containing 1 unit of precipitin per unit volume; x, the distance of the corresponding band formed by the undiluted, unknown serum; and a, the constant interval in millimeters between the positions of the bands formed by serial dilutions of the "standard" serum (usually 0.5 mm.). In each individual test, the values for x_s and a are checked by the inclusion of the two dilutions of "standard" serum. The distance between serum and virus cups in constant (10 mm.).

As an estimate of the variability inherent in the method, 95 per cent confidence limits were calculated for the major-band curve relating to the 50-fold virus concentrate (Text-fig. 1). The points on this curve are the mean values derived from four replicate titrations, and the small dots placed vertically above and below each point delimit the ± 2 S.D. interval. The points relating to the other three curves represent, not mean values, but individual readings.

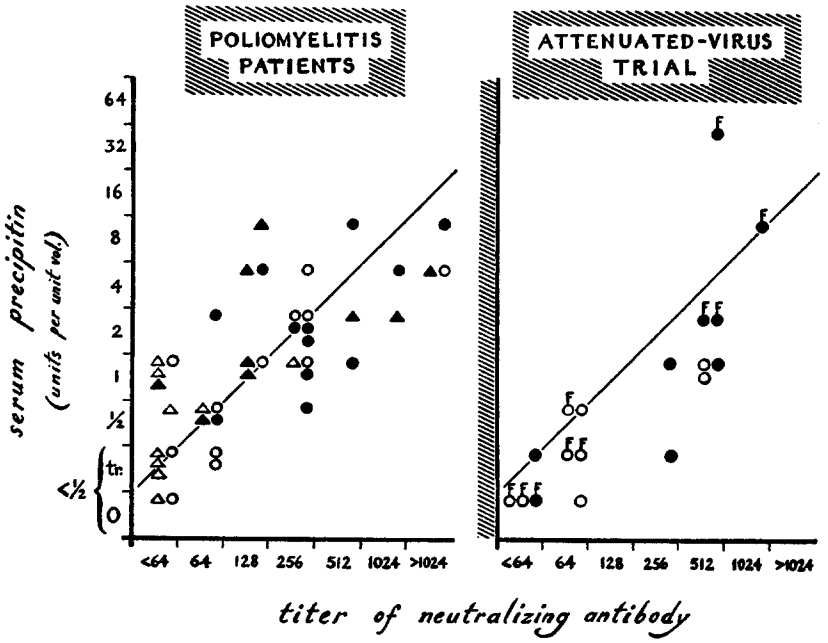
The thicker segments of the four curves indicate the range of serum dilutions found to be optimal for the three virus concentrations which can form a major band, and for the single concentration, the 50-fold, with which a minor band is observable. These optimal serum dilutions are those which produce, with the virus concentrations in question, the narrowest, sharpest, and densest band of precipitate. In these combinations of dilutions, the antigens and antibodies concerned are present in equivalent proportions.

The comparative antibody titers of the three "standard" monkey antisera, in order of type, were as follows: neutralizing, assayed by the panel test (25), — 2000, 500, and 8000; complement-fixing, against unheated virus, assayed by the plate test (22), — 120, 60, and >240; precipitating, — 8, 4, and 64. The titers of neutralizing and complement-fixing antibodies are expressed as the reciprocals of the end-point serum dilutions; the concentrations of precipitin, as units per unit volume of serum.

Determination of Poliovirus Precipitins in Human Serum

The test for assay of poliovirus precipitins in human serum is set up as illustrated in Fig. 3.

Four unknown sera can be tested on one plate; so that three plates suffice to test a pair of specimens from each of two patients against all three virus types. Virus, usually as the 50-



TEXT-FIG. 2. Comparative levels of poliovirus precipitins and homotypic neutralizing antibodies. The serum precipitin values, determined in plate agar diffusion tests, are calculated from the formula given in the text: tr. = a trace of precipitate; 0 = no precipitate detectable.

Poliomyelitis Patients.—Antibody titers against type of infecting virus in 21 paired sera, taken in 1st week (O, △) and 3rd to 6th week (●, ▲) of illness: circles indicate Type 1; triangles, Type 3.

Attenuated-Virus Trial.—Type 1 antibody levels in 9 individuals before (O), and 1 month after (●), the feeding of homotypic, attenuated virus (L-Sc strain). All 9 had antibody before the feeding: in 4, it had been naturally acquired; in the other 5, shown in the graph by the letter *F*, it had been produced by vaccination with formalin-treated virus.

fold concentrate, is placed in the larger, central cup. Two selected dilutions of reference serum are added to diagonally opposite cups, the higher dilution being placed between the cups containing the two acute-phase sera. The unknown sera are used undiluted. Fig. 3 shows the appearance, after 5 days at 35–37°C., of a Type 3 test on paired sera of two patients from whom Type 1 virus was isolated. In this example, the reference serum cups contain 4 and 16 pre-

cipitin units, respectively. The patient whose sera are in the upper half of the plate had no detectable Type 3 precipitins; the other patient, by contrast, possessed rather high, and virtually unchanging, levels of Type 3 precipitin (4 units per unit vol.), both early and late in his illness.

The relationship between poliovirus precipitins and homotypic neutralizing antibodies is depicted in Text-fig. 2.

The left-hand panel shows the results obtained on paired sera from 21 poliomyelitis patients, 12 of whom were infected with Type 1, and 9 with Type 3 virus. Only antibodies of the same type as that of the infecting virus are shown in the figure. Precipitins against virus types other than the one causing the infection were only found if the corresponding neutralizing antibodies were also present; and, in this group of patients, they only increased in amount in those whose neutralizing antibody titer did likewise.

TABLE I

Comparative Trends of Poliovirus Neutralizing and Precipitating Antibodies in the First Weeks of Poliomyelitis

Kind of antibody (of same type as that of infecting virus)	Type of poliovirus isolated from patient	Patients'* antibody trends between acute phase (1st week) and convalescent phase (3rd to 6th week) of illness			
		Decline	No change	Rise	
				2 ×	≥4 ×
Neutralizing	1	—	a, e, f, i, k, l	—	b, c, d, g, h, j
	3	—	m	r	n, o, p, q, s, t, u
Precipitating	1	f	j, k, l	g, i	a, b, c, d, e, h
	3	—	m	n, r	o, p, q, s, t, u

* Each individual patient is represented in the table by a given letter of the alphabet. 12 patients were infected with Type 1 poliovirus; 9, with Type 3.

From Text-fig. 2, a general correlation is apparent between the levels of neutralizing and precipitating antibody in any given specimen of serum taken during the first weeks of the disease. When the neutralizing antibody titer, estimated by the panel test (25), reaches a level of from 128 to 256, the precipitin content first becomes clearly measurable, with a value of 1 unit per unit volume of serum. Since their levels in individual serum specimens appear to be related, both kinds of antibody should show similar trends during this time. The comparative antibody trends in the same 21 patients of the text-figure are set out in Table I. As in Text-fig. 2, only antibodies of the infecting-virus type are there included. The trends of the two antibodies during the first weeks after the acute illness resemble each other quite closely: 13 of the 21 patients (62 per cent) show a 4-fold or greater rise in neutralizing antibody; while 12 (57 per cent) show a corresponding rise in precipitin of the

same type. Ten patients (48 per cent) are common to the two groups, and show rises in both kinds of antibody.

Too few tests have as yet been done to say whether precipitins go hand in hand with total, with "high avidity," or with "low avidity" neutralizing antibodies (*cf.* reference 33).

Whereas there appears to be a fairly close correlation between the content of (major-band) precipitins and that of homotypic "Y" complement-fixing antibodies detectable with unheated poliovirus antigens (7, 8), this does not apply to those antibodies ("H") that fix complement with heated antigens. It is possible that the crude 50-fold virus concentrates used in these tests do contain the appropriate antigen to form specific aggregates with "H," or with related, antibodies, but in quantities insufficient to give rise to visible precipitates.

The behavior of Types 2 and 3 precipitins resembles that of the Type 1, in relation to the homotypic neutralizing and complement-fixing antibodies.

In the right-hand panel of Text-fig. 2 are recorded the comparative levels of Type 1 neutralizing and precipitating antibody in the serum of 9 individuals before, and 1 month after, the ingestion of live, attenuated poliovirus, of the Type 1, L-Sc strain (27). Here, again, there is a tendency for the two kinds of antibody to go together; and they have continued to run parallel, in 3 of these 9 individuals, for at least 3½ months after the feeding of virus.

DISCUSSION

Poliovirus particles, or their derivatives, can be specifically aggregated by antibodies present in monkey hyperimmune and human convalescent serum, so as to form visible precipitates. Can this phenomenon serve to throw any light on the antigenic anatomy of the polioviruses? And do precipitating, as distinct from other kinds of antibodies, furnish any special information which might be of diagnostic value in human poliovirus infection?

As far as the composition of polioviruses is concerned, the precipitation reaction in agar has helped to distinguish between two antigenic constituents present in crude concentrates and in purified preparations.[6] Moreover, the technique has only been applied to virus-infected culture liquids concentrated by ultracentrifugation or by the method of physico-chemical precipitation, butanol extraction, enzymatic digestion, and centrifugation employed by Schwerdt and Schaffer (35). It is possible that other methods of concentration, such as ultrafiltration or pervaporation, might retain less easily sedimentable antigenic material possessing yet other reactivity.

[6] For purified fractions, there is evidence, from studies undertaken at the University of California, Berkeley, in collaboration with Dr. C. E. Schwerdt and Dr. F. L. Schaffer, that the two precipitating components differ not only physically, with respect to their sedimentation constants and the morphology of their associated particles, but also chemically, in the sense of being qualitatively distinct antigens (34).

The results of experiments not here reported indicate that the character of a specific poliovirus precipitate may be altered, and its amount reduced, by preheating the virus concentrate at 60°C. for 20 minutes, or by the addition of merthiolate to the agar in final concentrations ranging from 0.1 per cent down to 0.001 per cent *w/v*. The effects produced by these agents vary in degree with virus strains of different type. Since heat and merthiolate are known to impair the capacity of polioviruses to stimulate protective antibodies, there may prove to be a useful, practical link between the effective immunogenicity of polioviruses and their activity as precipitating antigens. In this connection, it may be worth noting that treatment with 0.01 per cent formaldehyde (final concentration) for 3 days at 37°C. was relatively less detrimental to precipitate formation than either heat or merthiolate, although here, too, the effect of treatment appeared to vary with the strain of virus.

As to the value of precipitin estimations in poliovirus infections, it must be said that there seems, at present, to be no idiosyncratic attribute of the precipitating antibodies which might be turned to good account in diagnostic serology. They exhibit a general correlation with neutralizing antibodies, and it may reasonably be inferred that many antibody molecules are capable of taking part in both kinds of reaction; but there is, as yet, no suggestion that precipitating activity runs parallel with neutralizing capacity of high, rather than with that of low, "avidity." More information is needed on this point.

Probably the outstanding, practical advantage of those immunological reactions which depend on the formation of visible aggregates is their speed. It is possible, therefore, that a direct precipitin test with liquid reactants may deserve a place in the detection of poliovirus infection. Wilson Smith and his colleagues have described such a test (19, 20); and the ordinary "ring" test can be useful. Both these methods demand reactants of fair clarity; but if this often burdensome condition can be met, then a rapid precipitin test on an acute-phase serum may possibly acquire a certain value. As a provisional judgment, it seems unlikely that precipitin tests in agar would establish themselves as regular diagnostic procedures, even if adequate supplies of virus concentrates were to become available. Yet the advantages of quantitative plate tests in permitting a direct, visual comparison of the precipitin content of paired, or serial, specimens of serum may recommend their use in special investigations, in which a knowledge of the whole range of recognized poliovirus antibodies is desired.

SUMMARY

Antigens present in poliovirus concentrates react with antibodies present in the serum of hyperimmunized monkeys to give type-specific precipitates. One or more bands of precipitate can be formed wherever such homotypic reactants,

diffusing into an agar gel, meet in sufficient concentration and in equivalent proportions.

No qualitative differences have been detected between the type-specific reactions given by different strains of the same virus type; and no precipitates have been seen which could be called "group-specific." Non-specific precipitates have occasionally been observed.

Type-specific poliovirus precipitins are found in the serum of poliomyelitis patients. Their concentration has been measured by a standardized method. They tend to develop in parallel with neutralizing antibodies against the same virus type, increasing in amount during the first weeks of illness in those patients who show concurrent neutralizing antibody rises.

The precipitation reaction has proved valuable in the antigenic analysis of polioviruses. Its general adoption as a diagnostic procedure is perhaps unlikely; but it may be well worth applying for special purposes.

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EXPLANATION OF PLATES

Key to figures.—

1, poliovirus Type 1 (Brunhilde)
2, poliovirus Type 2 (Y-SK)
3, poliovirus Type 3 (Leon)
3ª, poliovirus Type 3 (Saukett)
C, Coxsackie virus Type A9 (Grigg)
} 50-fold concentrates

i, Type 1 (Brunhilde)
ii, Type 2 (Y-SK)
iii, Type 3 (Leon)
} Monkey hyperimmune antisera

a,*a'*, patients' sera, taken in 1st week of illness
b,*b'*, patients' sera, taken in 3rd and 6th week of illness, respectively
All reactants are unheated and undiluted, unless otherwise indicated.

PLATE 90

FIG. 1. Titration of poliovirus precipitins by the method of one dimensional, double diffusion in agar. Serial, twofold dilutions of a Type 1 (Brunhilde) monkey antiserum are tested against a 50-fold concentrate of Type 1 (Mahoney) poliovirus. Photograph taken after the tubes had been held for 7 days at 4°C. With increasing dilution of the serum, the two bands of precipitate ("major" and "minor") become progressively fainter and develop closer to the serum depot. × 1.3.

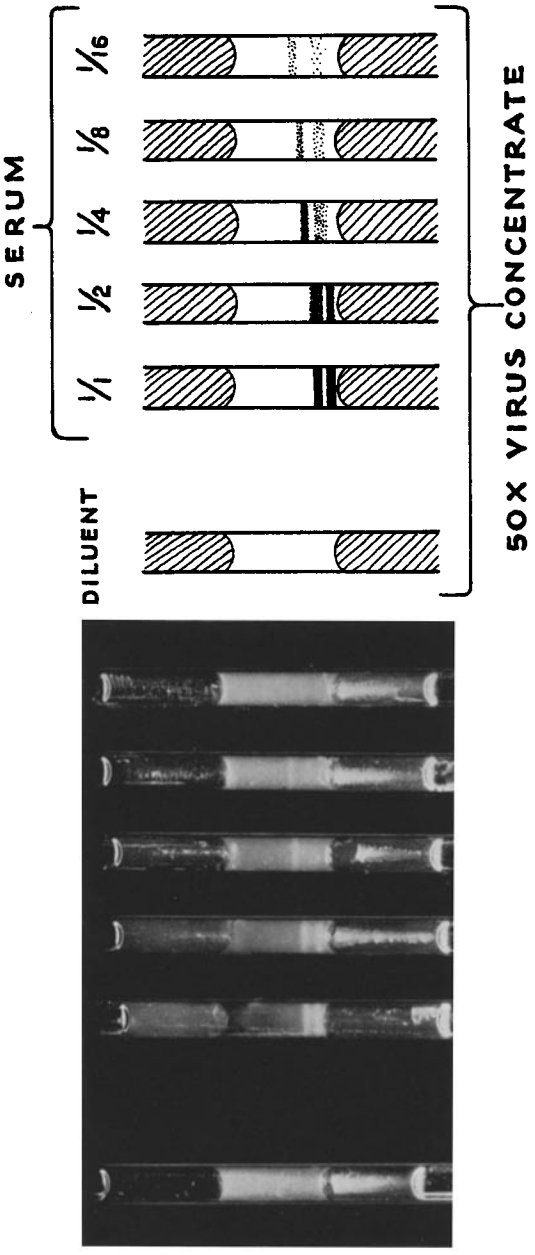

FIG. 1

(Le Bouvier: Poliovirus precipitation in agar)

PLATE 91

FIG. 2. Type specificity of the precipitation reaction between polioviruses and homotypic monkey antisera, both diffusing into agar in a two dimensional, plate test. Photograph taken after the plate had been kept at 4°C. for 22 days, during which time the cups were refilled with reactant at 5-day intervals. Certain of the precipitate bands have been formed only between cups containing virus and serum of the same type. × 1.4.

FIG. 3. Type 3 poliovirus precipitin test, in agar, on acute- and convalescent-phase sera of two poliomyelitis patients who had Type 1 infections. Photograph taken after the test plate had been incubated for 5 days at 35–37°C. Both negative and positive results are seen. The upper pair of sera (*a*, *b*) gives no visible precipitate with the Type 3 virus concentrate. But the lower pair (*a'*, *b'*) forms clear bands, which fuse with those produced by the two dilutions of the "standard" Type 3 monkey antiserum, included in the test for purposes of assay. The similarity, in appearance and position, between the precipitates formed by serum *a'* and serum *b'* indicates that they possess comparable levels of Type 3 poliovirus precipitin. × 1.4.

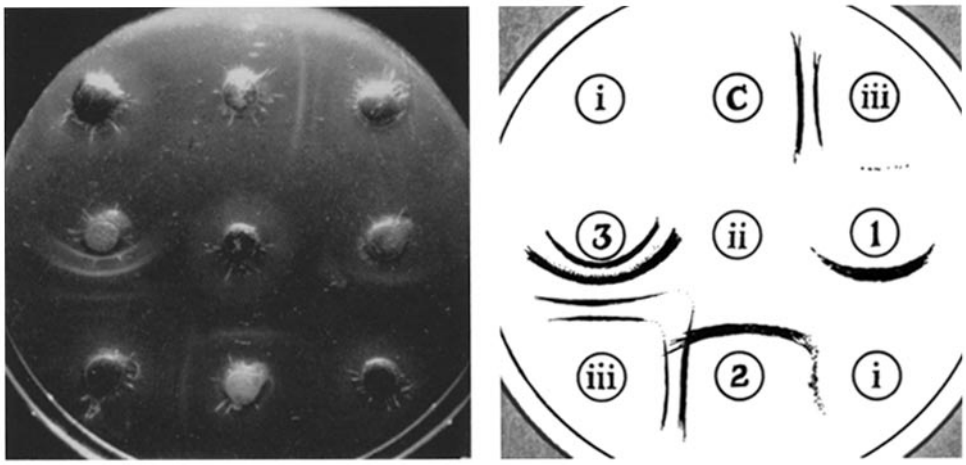

FIG. 2

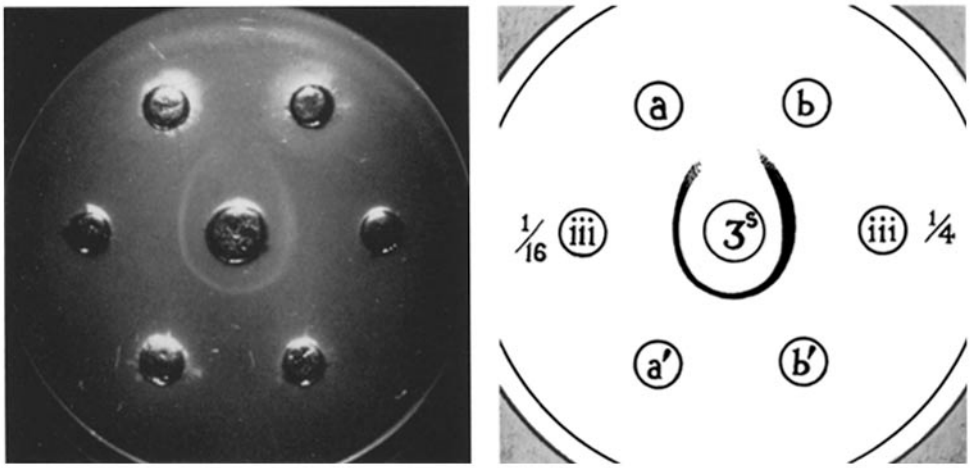

FIG. 3

(Le Bouvier: Poliovirus precipitation in agar)