MODIFICATION OF THE HOMOTYPIC SPECIFICITY OF POLIOMYELITIS COMPLEMENT-FIXING ANTIGENS BY HEAT*

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In a recent communication (1) we described a simple, rapid, and practical complement fixation method for the diagnosis of poliomyelitis. The procedure, which employs *fully infectious fluid* from tissue cultures in which the poliomyelitis viruses have been grown, was found to have a very high degree of homotypic specificity. To encourage the use of the procedure in diagnostic laboratories unfamiliar with the handling of viruses, it is highly desirable to remove the infection hazard by destroying the infectivity of the antigens without affecting their complement-fixing activity, and it was thought that this might be achieved by heating. This report deals with comparative studies[1] on heated and unheated antigens and shows (*a*) that the difficulties with heterotypic fixation reported in the literature are attributable in large part to the use of heated poliomyelitis complement-fixing antigens and (*b*) that heat treatment uncovers a reactive fraction common to the three virus types.

Material and Methods

Poliomyelitis Virus Strains Used.—For the preparation of complement-fixing antigens the Mahoney (Type 1), Statler (Type 2), and McMullen (Type 3) strains were used. The Statler and McMullen strains were isolated in this laboratory and were used because they appeared to give slightly better antigens than did the MEF-1 (Type 2) and Saukett (Type 3) strains.

For the *in vitro* neutralization tests, the Mahoney, the MEF-1, and Saukett strains were used.

Preparation of Complement-Fixing Antigens.—Of the several lines of HeLa cells in this laboratory, only the D and the X lines[2] are used for the preparation of poliomyelitis complement-fixing antigens. The HeLa cells are routinely carried in square 200 ml. milk dilu-

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[1] The report of LeBouvier (2) on the modification of polio virus antigens by heat and ultraviolet light appeared while this work was in progress, and his findings as they relate to the present work will be discussed when indicated.

[2] These cell lines were obtained from Microbiological Associates, Bethesda.

tion bottles on an outgrowth medium consisting of 10 per cent human serum and 90 per cent lactalbumin hydrolysate–yeast extract.[3]

Cells to be used for the production of complement-fixing antigen are seeded into a bottle containing an outgrowth medium composed of 90 per cent lactalbumin hydrolysate–yeast extract medium to which 10 per cent lamb serum is added rather than the human serum, as it has been found that cells grown in this medium adhere to the glass better than do cells carried exclusively on human serum.

Antigens were prepared according to the following procedure.

Milk dilution bottles (200 ml.) were seeded with 1×10^6 HeLa cells suspended in 10 ml. of lactalbumin hydrolysate–yeast extract medium containing 10 per cent lamb serum. After incubation at 36°C. for 2 days, 5 ml. of fresh medium was added to that already in the bottles. At 5 days, all of the medium was replaced by 10 ml. of fresh medium. At 7 days, 5 ml. of fresh medium was added to each bottle, and at 8 days all of the medium was removed and replaced with 10 ml. of fresh medium.

Specific antigens were made from cell cultures inoculated with virus on the 9th day. At this time the number of cells in each bottle ranged between 15×10^6 and 20×10^6. The outgrowth medium was removed and the cell sheets were washed twice with Hanks's balanced salt solution. After the washing, 8 ml. of maintenance medium, consisting of 2.5 per cent normal inactivated monkey serum and 97.5 per cent of medium 199 in Earle's solution was added to each bottle, followed by 0.5 ml. of a 1×10^{-1} dilution of tissue culture passage virus.

The inoculated cultures were incubated for 3 days at 36°C. The infected fluids were harvested, pooled by virus strain, ampouled in 10 ml. amounts and stored at −20°C. When titrations for antigenic activity were performed, or material was required for testing sera, the contents of the requisite number of ampoules were thawed, centrifuged horizontally at 2,000 R.P.M. for 15 minutes, and the supernatant fluid drawn off for use.

Control antigens consisted of fluids harvested from uninoculated HeLa cell cultures which had been handled as outlined above for the infected cultures, including incubation for 3 days at 36°C. These fluids served as controls for the detection of possible non-specific complement-fixing activity and in the test proper were used in 0.2 ml. amounts of a 1:2 dilution.

Heated antigens consisted of infected or uninfected fluids heated at 56°C for 30 minutes in a water bath. These antigens were never prepared as separate lots of material, but always consisted of an aliquot of a lot of unheated antigen. During heating the container was submerged so that the level of the fluid within it was below that of the water level of the bath; the inactivation time was counted from the time when the thermometer in the container indicated that the contents had reached 56°C.

In Vitro Neutralization Tests.—Both monkey kidney and HeLa cells were used.

The colorimetric method described by Salk, Youngner, and Ward (3), employing monkey kidney cells, was used to determine the neutralizing antibody level of certain sera, and the colorimetric method of Lipton and Steigman (4) based on the use of HeLa cells, was used with other sera. The technic employed is indicated in the tables.

Titration of Antigens.—The potency of each antigen preparation was determined in "checkerboard" or "block" titrations with hyperimmune monkey serum; in this procedure, falling dilutions of the antigen are tested against falling dilutions of the immune serum. The titration endpoint which represents 1 unit of antigen is defined as that dilution of antigen which gives three plus or four plus fixation with the highest dilution of immune serum.

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[3] The lactalbumin hydrolysate–yeast extract medium has the following composition: Lactalbumin hydrolysate, 0.5 per cent; glucose, 0.45 per cent; yeast extract, 0.1 per cent; and sodium bicarbonate, 0.11 per cent.

These components are dissolved in Earle's balanced salt solution.

In the complement fixation test proper, 2 units of antigen contained in 0.2 ml. were used.

Complement.—Lyophilized complement was used throughout this work; it was reconstituted in the fluid supplied by the manufacturer. Complement was titrated in the presence of each specific antigen and two *exact* units in a volume of 0.2 ml. were used in the tests. The incubation period for the titration was 1 hour at 37°C.

Hemolysin.—Two units of hemolysin in 0.25 ml. as determined by titration at 37°C. for 30 minutes with 1:30 complement (0.2 ml.) and a 2.0 per cent sheep cell suspension (0.25 ml.) were used.

Sensitized Cells.—Sensitized cells were prepared by mixing equal volumes of a 2 per cent suspension of sheep erythrocytes and a dilution of hemolysin containing 2 hemolytic units in a volume of 0.25 ml. The mixture was allowed to stand at room temperature for 10 minutes before use.

Procedure for the Complement Fixation Test.—Sera were diluted 1:4 in Kolmer saline solution and inactivated at 60°C. for 30 minutes. (Occasionally, because the amount of serum available was inadequate to permit a dilution series beginning at 1:4, the specimen was diluted 1:8 before inactivation.) After inactivation, serial 2-fold dilutions of each serum were prepared in Kolmer saline solution. Each dilution was prepared in adequate amounts to permit dispensing into a series of tubes so that tests against the various antigens could be run concurrently. In addition, the first two dilutions of each serum were tested for anticomplementary activity in the absence of antigen, and for possible non-specific complement-fixing activity with control antigens. Antigen diluted to contain 2 units in a volume of 0.2 ml. was added, followed by 0.2 ml. of complement dilution containing 2 exact units. Fixation was allowed to proceed at 4°C. overnight (approximately 18 hours). The tubes were then warmed in a 37°C. water bath for 10 minutes, 0.5 ml. of sensitized cells was added to each tube, and secondary incubation was conducted for 15 to 30 minutes, depending upon the time required for the complement control tubes to show clearing. The complement controls consisted of a series of four tubes containing antigen, sensitized red cells, and 2 units, 1½ units, 1 unit, and ½ unit of complement, respectively. The tubes containing 1, 1½, and 2 units should show clearing after 15 to 30 minutes of incubation, and as soon as this occurred the tests were read. The titer of a serum was the highest dilution (initial, not final) showing three plus or four plus fixation with the specific antigen.

Monkey Hyperimmune Serum.—Specific monovalent immune sera for use in the complement fixation and *in vitro* neutralization tests were prepared in *rhesus* monkeys. The Mahoney, MEF-1, and Saukett strains were used. The animals were inoculated into the calf muscles at biweekly intervals for a total of three or four inoculations with 2 ml. of a mixture of adjuvant and infected fluid from monkey kidney tissue cultures of the virus. The adjuvant consisted of one part of arlacel A in nine parts of standard mineral oil C.T. 70.[4] Equal parts of the adjuvant mixture and culture fluid were emulsified by vigorous shaking in a vaccine vial containing glass beads.

The hyperimmune serum prepared in this manner was type-specific in the complement fixation and neutralization tests. The complement-fixing antibody titers of the sera against the homologous antigens were usually 1:1024 and the neutralizing titers in metabolic inhibition tests were 1:16,384 or higher.

RESULTS

Effect of Heat on Complement-Fixing Potency and Sensitivity of the Antigens.—

Svedmyr *et al.* (5, 6) reported that their tissue culture antigens had to be concentrated to give usable preparations, and that concentration resulted in the appearance

[4] Arlacel was generously provided by the Atlas Powder Company, Wilmington. Standard mineral oil C.T.70 is a product of the Standard Oil Company of California, Richmond.

of anticomplementary activity. Black and Melnick (7) also encountered difficulties with the anticomplementary proclivities of some of their tissue culture antigens. Both groups found that this undesirable property could be abolished by heat treatment (54°C. to 60°C. for 30 minutes (5) or 56°C. for 30 minutes (7)) and that the ability of the antigens to fix complement in the presence of homotypic monkey immune serum was not destroyed (5, 6, 8). Black (9) states that heating the virus at 56°C. for 30 minutes destroys the infectivity without significantly reducing or broadening the antigenicity.

As quantitative data were not available on the possible adverse effects of heat on the sensitivity and potency of tissue culture fluids used as antigens, a comparison of heated and unheated antigens was made with respect to these properties. Four different lots of Type 1 antigen, six of Type 2, and three of Type 3 were assayed. An aliquot of each antigen was heated, and the heated and unheated preparations were examined concurrently with homotypic monkey immune serum. The results are given in Tables I, II and III, in which the titration endpoints (antigenic units) are indicated.

That heating quantitatively affects the potency of the antigens is illustrated in Table I, which shows that all four lots of the Type 1 antigen suffered a reduction in fixing capacity after heat treatment. The antigenic titer of antigen 6199 was lowered from 1:8 to 1:6; of antigen 6195, from 1:6 to 1:4; of antigen 5033, from 1:6 to 1:3, and of antigen 5388, from 1:4 to 1:3. While, at first glance, the reduction in titer may appear quantitatively insignificant, it is relatively large. Thus, heating preparations 6199 and 5388 resulted in a 25 per cent loss in fixing capacity and heating of antigens 6195 and 5033 resulted in a 30 per cent and 50 per cent loss in potency respectively. On the applied side, losses in fixing capacity such as a loss encountered with antigen 5033 would necessitate twice the amount of heated antigen to do the same number of examinations possible with unheated material.

In addition to the loss of antigenic potency with heating, there was a concomitant loss in sensitivity. For example, antigens 6199 and 6195, which when used undiluted gave a serum endpoint titer of 1:2048, gave a titer of only 1:512 after heating. This is also illustrated by the fact that, if the antigenic unit is taken as the endpoint of the titration, 1 unit of unheated antigen consistently gave fixation with one 2-fold higher dilution of immune serum than did the headed antigen. These findings agree with those just recently reported by LeBouvier (2).

The results of comparative titrations of heated and unheated Type 2 complement-fixing antigens of six different lots are given in Table II. The potency of five of the six antigens was reduced by heating. The effects of heating were most marked in antigen 6350, whose titer (antigenic unit) dropped from 1:8 to 1:3. The titer of antigen 6349 decreased from 1:8 to 1:4 and that of antigens 6114, 6334, and 6335 decreased from 1:4 to 1:3. The antigenic titer of antigen 6340

TABLE I

Comparison of Potency and Sensitivity of Heated and Unheated Type 1 Poliomyelitis Virus Complement-Fixing Antigens Tested with Homologous Monkey Immune Serum

Dilution of Antigen	Results obtained with:									
	Unheated antigen					Heated antigen				
	Dilutions of immune serum					Dilutions of immune serum				
	1:128	1:256	1:512	1:1024	1:2048	1:128	1:256	1:512	1:1024	1:2048
Antigen 6199										
Undiluted	4+	4+	4+	4+	3+	4+	4+	4+	0	0
1:2	4+	4+	4+	4+	2+	4+	4+	4+	0	0
1:3	4+	4+	4+	4+	1+	4+	4+	4+	0	0
1:4	4+	4+	4+	4+	0	4+	4+	4+	0	0
1:6	4+	4+	4+	4+	0	4+	4+	4+/	0	0
1:8	4+	4+	4+	3+/*	0	4+	4+	2+	0	0
1:10	4+	4+	2+	0	0	0	0	0	0	0
	Titer = 1:8					Titer = 1:6				
Antigen 6195										
Undiluted	4+	4+	4+	4+	3+	4+	4+	4+	0	0
1:2	4+	4+	4+	4+	0	4+	4+	4+	0	0
1:3	4+	4+	4+	4+	0	4+	4+	4+	0	0
1:4	4+	4+	4+	4+	0	4+	4+	4+/	0	0
1:6	4+	4+	4+	4+/	0	4+	4+	1+	0	0
1:8	4+	4+	3+	0	0	1+	0	0	0	0
1:10	4+	2+	0	0	0	0	0	0	0	0
	Titer = 1:6					Titer = 1:4				
Antigen 5033										
Undiluted	4+	4+	4+	3+	0	4+	4+	4+	2+	0
1:2	4+	4+	4+	1+	0	4+	4+	4+	2+	0
1:3	4+	4+	4+	0	0	4+	4+	4+/	0	0
1:4	4+	4+	4+	0	0	4+	4+	0	0	0
1:6	4+	4+	4+/	0	0	4+	4+	0	0	0
1:8	4+	2+	0	0	0	4+	2+	0	0	0
	Titer = 1:6					Titer = 1:3				
Antigen 5388										
Undiluted	4+	4+	4+	3+	0	4+	4+	4+	1+	0
1:2	4+	4+	4+	4+	0	4+	4+	4+	0	0
1:3	4+	4+	4+	3+	0	4+	4+	3+/	0	0
1:4	4+	4+	4+	3+/	0	4+	4+	±	0	0
1:6	4+	4+	1+	0	0	4+	1+	0	0	0
1:8	0	0	0	0	0	0	0	0	0	0
	Titer = 1:4					Titer = 1:3				

* __/ = Antigenic endpoint of titration.

Antigens prepared from Mahoney strain, Type 1 virus.

Immune sera prepared against Mahoney strain, Type 1 virus.

TABLE II

Comparison of Potency and Sensitivity of Heated and Unheated Type 2 Poliomyelitis Virus Complement-Fixing Antigens Tested with Homologous Monkey Immune Serum

Dilution of antigen	Results obtained with:									
	Unheated antigen					Heated antigen				
	Dilutions of immune serum					Dilutions of immune serum				
	1:128	1:256	1:512	1:1024	1:2048	1:128	1:256	1:512	1:1024	1:2048
Antigen 6350										
Undiluted	4+	4+	4+	4+	2+	4+	4+	4+	2+	0
1:2	4+	4+	4+	4+	0	4+	4+	4+	2+	0
1:3	4+	4+	4+	4+	0	4+	4+	4+/0	0	0
1:4	4+	4+	4+	4+	0	4+	4+	0	0	0
1:4	4+	4+	4+	4+	0	4+	4+	0	0	0
1:6	4+	4+	4+	4+	0	0	0	0	0	0
1:8	4+	4+	4+	3+/0*	0	0	0	0	0	0
1:10	4+	3+	0	0	0	0	0	0	0	0
	Titer = 1:8					Titer = 1:3				
Antigen 6349										
Undiluted	4+	4+	4+	4+	2+	4+	4+	0	0	0
1:2	4+	4+	4+	4+	2+	4+	4+	±	0	0
1:3	4+	4+	4+	4+	1+	4+	3+	±	0	0
1:4	4+	4+	4+	4+	0	4+	3+/2+	0	0	0
1:6	4+	4+	4+	4+	0	4+	2+	0	0	0
1:8	4+	4+	4+	4+/0	0	4+	1+	0	0	0
1:10	4+	2+	0	0	0	0	0	0	0	0
	Titer = 1:8					Titer = 1:4				
Antigen 6340										
Undiluted	4+	4+	4+	0	0	4+	4+	0	0	0
1:2	4+	4+	4+	0	0	4+	4+	0	0	0
1:3	4+	4+	4+	0	0	4+	4+	0	0	0
1:4	4+	4+	4+/0	0	0	4+	4+/2+	0	0	0
1:6	4+	0	0	0	0	4+	2+	0	0	0
1:8	0	0	0	0	0	0	0	0	0	0
	Titer = 1:4					Titer = 1:4				
Antigen 6335										
Undiluted	4+	4+	4+	0	0	4+	4+	4+	0	0
1:2	4+	4+	4+	0	0	4+	4+	4+	0	0
1:3	4+	4+	4+	0	0	4+	4+	3+/0	0	0
1:4	4+	4+	3+/0	0	0	4+	4+	0	0	0
1:6	0	0	0	0	0	4+	3+	0	0	0
1:8	0	0	0	0	0	0	0	0	0	0
	Titer = 1:4					Titer = 1:3				

TABLE II—*Concluded*

Dilution of antigen	Results obtained with:									
	Unheated antigen					Heated antigen				
	Dilutions of immune serum					Dilutions of immune serum				
	1:128	1:256	1:512	1:1024	1:2048	1:128	1:256	1:512	1:1024	1:2048
Antigen 6114										
Undiluted	4+	4+	4+	1+	0	4+	4+	0	0	0
1:2	4+	4+	4+	0	0	4+	4+	0	0	0
1:3	4+	4+	4+	0	0	4+	3+/	0	0	0
1:4	4+	4+	4+/	0	0	4+	2+	0	0	0
1:6	4+	3+	2+	0	0	0	0	0	0	0
1:8	4+	0	0	0	0	0	0	0	0	0
	Titer = 1:4					Titer = 1:3				
Antigen 6334										
Undiluted	4+	4+	4+	2+	0	4+	4+	0	0	0
1:2	4+	4+	4+	±	0	4+	4+	0	0	0
1:3	4+	4+	4+	0	0	4+	4+/	0	0	0
1:4	4+	4+	4+/	0	0	3+	±	0	0	0
1:6	4+	3+	0	0	0	0	0	0	0	0
1:8	±	0	0	0	0	0	0	0	0	0
	Titer = 1:4					Titer = 1:3				

* _/ = Antigenic endpoint of titration.
Antigen prepared from Statler strain, Type 2 virus.
Immune sera prepared against MEF-1 strain, Type 2 virus.

was 1:4 both before and after heating. With the exception of antigen 6335, there was also a lowering of the serum endpoint titer. In four of the antigens, *viz.*, 6114, 6334, 6340, and 6350, the serum titer was decreased by one 2-fold dilution but in antigen 6394 it was decreased by two 2-fold dilutions; *i.e.*, from 1:1024 to 1:256.

Three different lots of Type 3 antigen were tested. Interestingly enough, heating did not affect the antigenic titer of any of the three lots, although as inspection of Table III will show, sensitivity was affected just as in the case of the Type 1 and Type 2 antigens.

Some of our findings on the effect of heat agree with those recently reported by LeBouvier (2) while others apparently do not. LeBouvier reported that the degree of fixation obtained with heated antigen (60°C. for 20 minutes) and homotypic monkey immune sera was regularly less than that with the unheated antigen. If the Type 3 strain used is representative of Type 3 strains in general, the question arises as to why the Type 3 antigen was essentially unaffected by heating, whereas such treatment of the Type 1 and Type 2 antigens resulted in a loss of antigenic potency. The heat

TABLE III

Comparison of Potency and Sensitivity of Heated and Unheated Type 3 Poliomyelitis Virus Complement-Fixing Antigens Tested with Homologous Monkey Immune Serum

Dilution of antigen	Results obtained with									
	Unheated antigen					Heated antigen				
	Dilutions of immune serum					Dilutions of immune serum				
	1:128	1:256	1:512	1:1024	1:2048	1:128	1:256	1:512	1:1024	1:2048
Antigen 6200										
Undiluted	4+	4+	4+	4+	2+	4+	4+	4+	3+	0
1:2	4+	4+	4+	4+	1+	4+	4+	4+	±	0
1:3	4+	4+	4+	4+	±	4+	4+	4+	0	0
1:4	4+	4+	4+	4+	0	4+	4+	4+	0	0
1:6	4+	4+	4+	4+	0	4+	4+	4+	0	0
1:8	4+	4+	4+	3+/*	0	4+	4+	4+/	0	0
1:10	4+	3+	2+	0	0	4+	2+	0	0	0
	Titer = 1:8					Titer = 1:8				
Antigen 5708										
Undiluted	4+	4+	4+	4+	0	4+	4+	4+	0	0
1:2	4+	4+	4+	4+	0	4+	4+	4+	0	0
1:3	4+	4+	4+	4+	0	4+	4+	4+	0	0
1:4	4+	4+	4+	4+	0	4+	4+	4+	0	0
1:6	4+	4+	4+	3+/	0	4+	4+	3+/	0	0
1:8	4+	0	0	0	0	0	0	0	0	0
	Titer = 1:6					Titer = 1:6				
Antigen 6324										
Undiluted	4+	4+	4+	4+	±	4+	4+	4+	2+	0
1:2	4+	4+	4+	4+	0	4+	4+	4+	1+	0
1:3	4+	4+	4+	4+	0	4+	4+	4+	0	0
1:4	4+	4+	4+	4+/	0	4+	4+	4+/	0	0
1:6	4+	3+	2+	0	0	4+	4+	2+	0	0
1:8	±	0	0	0	0	4+	0	0	0	0
1:10	0	0	0	0	0	0	0	0	0	0
	Titer = 1:4					Titer = 1:4				

* _/ = Antigenic endpoint of titration.
Antigens prepared from McMullen strain, Type 3 virus.
Immune sera prepared against Saukett srain, Type 3 virus.

resistance of the Type 3 antigen does not accord with LeBouvier's observations, as he found that in conversion of poliomyelitis antigens from the Y (unheated) to the H (heated) form, the Type 3 virus was the most susceptible of the three types. He noted that "above 60°C, there was a diminution in the complement-fixing activity of the

poliovirus antigens over different ranges of temperature, the Type 3 virus ceasing to react with its 'standard' monkey antiserum after 20 minutes at 70°C., the Type 2 at 80°C., and the Type 1 at 90°C."

Reactivity of Heated Antigens with Heterotypic Monkey Immune Sera.—The effect of heat on the homotypic specificity of the antigen was next investigated.

Heating of poliomyelitis complement-fixing antigens has been a routine or frequent practice to abolish either infectivity or anticomplementary action (5–11). Svedmyr *et al.* (5, 6) reported that their antigens (heated) gave no cross-reactions with heterotypic monkey immune sera, and Black and Melnick (12) stated that their heated

TABLE IV

Reactivity with Monkey Immune Sera of Heated and Unheated Poliomyelitis Virus Complement-Fixing Antigens

Monkey No.	Immune serum	Complement-fixing titer of immune serum with					
		Unheated antigen of			Heated antigen of		
		Type 1	Type 2	Type 3	Type 1	Type 2	Type 3
1	Type 1 (Mahoney)	1024	<8	<8	512	<8	<8
2	Type 1 (Mahoney)	1024	<8	<8	256	<8	<8
3	Type 2 (MEF-1)	<8	2048	<8	<8	1024	<8
4	Type 2 (MEF-1)	<8	2048	<8	8	512	8
5	Type 3 (Saukett)	<8	<8	512	<8	<8	512
6	Type 3 (Saukett)	<8	<8	256	<8	<8	128

Antigens prepared from the Mahoney, Statler, and McMullen strains of Types 1, 2, and 3 viruses, respectively.

preparations were not cross-reactive with monkey immune serum. However, since the complement-fixing method used by the workers in the references just cited is a plate or micro method (13), information was desired on whether heated antigens would show heterotypic reactivity in the macro method (tube test) that we described (1).

Table IV summarizes briefly the results obtained when heated antigens were tested with heterotypic monkey immune sera. Each serum was examined in the "line test" in which serial 2-fold dilutions of serum were tested with 2 units of antigen, as determined by checkerboard titrations. It will be observed that the unheated antigens gave sharp, homotypic fixation—no cross-reactions between virus types occurred in these experiments. After heat treatment, the Type 2 antigen still showed completely homotypic activity. The heated Type 1 and Type 3 antigens, however, appeared to give a small degree of fixation with the Type 2 antiserum from monkey 4. The significance, if any, of this one discrepant result is not assessable at the moment, especially since fixation was at

threshold levels. However, the virtually complete absence of heterotypic activity on the part of the heated antigens in the tube complement fixation test is in substantial agreement with the findings of others (6, 12) using the plate method. Why heated antigens should relatively uniformly give sharply homotypic fixation with monkey immune sera but react heterotypically with human sera (see next section) is unclear.

Recent observations indicate that some modification of the antigenic structure occurs on exposure of the virus to formalin (7), heat (2), and ultraviolet irradiation (2). We (unpublished data) and others (7) have observed that immune sera from monkeys inoculated with monovalent, formalinized virus preparations react heterotypically in the complement fixation test. Black and Melnick (12) recently reported that tissue culture antigens, with sharply homotypic reactivity in their native state, became cross-reactive after treatment with formalin and suggested as one possibility that the broadened reactivity might be caused by exposure of a previously masked group antigen. LeBouvier (2) found that monkeys inoculated with heated virus developed antibodies which reacted to a higher titer with heated than with unheated antigens, while monkeys inoculated with unheated, fully active virus developed antibodies which reacted to a higher titer with unheated than with heated antigens. The reactions were primarily homotypic with occasional small degrees of cross-reaction, serum titers of 1:1 or up to 1:8 being shown by some of the test antisera with heterotypic complement-fixing antigens; cross-reactions were greater with heated than with unheated heterotypic antigens.

Effect of Heat on Homotypic Specificity of Antigens Used for Antibody Assay of Human Sera.—In a previous communication (1), we presented data illustrating the high degree of type-specific fixation encountered by us with human sera. It was considered possible that the relative freedom from cross-reactions might be due to the use of untreated, fully infectious antigens since others, who have reported a high degree of heterotypic reactivity, have used heated antigens (5–11). Svedmyr (10), for example, recently reported that in a series of 137 cases of poliomyelitis, the complement fixation test was positive in 90 per cent, but only 10 per cent showed type-specific reactions. Black (9), however, states that heating does not significantly broaden the antigenicity.

As no specific information was available in the literature as to the basis for these heterotypic reactions, a quantitative comparison of heated and unheated antigens was undertaken.

Each batch of antigen, heated or unheated, was titrated to determine the antigenic unitage, and line tests were done using 2 units of antigen and falling 2-fold dilutions of serum. Fifty sera were selected from a group on which neutralization tests had already been done, so as to include in the experiments sera with no demonstrable neutralizing antibody to one or another virus type, to two types, etc.

The results with the 50 sera are presented in Table V, in which the sera are arranged in order of increasing complement-fixing antibody to the Type 1 virus as determined by the use of unheated antigen.

TABLE V

Effect of Heat on Homotypic Specificity of Poliomyelitis Complement-Fixing Antigens Used for Antibody Determinations of Human Sera

Serum		Poliomyelitis C-F titer of serum with						Poliomyelitis-neutralizing antibody titer		
		Unheated antigen of			Heated antigen of					
Series No.	Accession No.	Type 1	Type 2	Type 3	Type 1	Type 2	Type 3	Type 1	Type 2	Type 3
1	54/712	<4	<4	<4	<4	<4	<4	<4	<4	16
2	55/55	<4	<4	<4	<4	<4	<4	<4	<4	128
3	1312	<4	<4	4	<4	<4	4	<4	<4	256
4	6945	<4	<4	8	<4	4	16	<4	<4	128
5	7920	<4	<4	8	8	16	8	<4	<4	128
6	1027	<4	<4	16	4	4	4	64	<4	128
7	7976	<4	<4	32	4	32	32	<4	<4	512
8	6581	<4	<4	32	8	64	64	<4	<4	1024
9	5895	<4	<4	64	16	32	16	64	16	512
10	5725	<4	<4	64	64	64	64	<4	<4	2048
11	7026	<4	<4	64	64	64	64	<4	<4	1024
12	6522	<4	16	4	<4	32	4	<4	256	4
13	1543	<4	16	16	<4	16	<4	<4	1024	128
14	2882	4	<4	<4	<4	16	<4	128	<4	<4
15	6521	4	<4	4	4	8	4	256	8	<4
16	167	8	<4	<4	<4	<4	<4	256	<4	<4
17	6406	8	<4	<4	4	<4	<4	256	<4	<4
18	1007	8	<4	<4	8	4	4	128	<4	<4
19	592	8	<4	<4	8	8	4	512	<4	<4
20	7629	8	<4	<4	64	32	32	1024	<4	<4
21	7242	8	8	<4	8	8	<4	64	512	<4
22	1336	8	16	<4	<4	4	<4	256	512	8
23	497	8	<4	4	8	16	4	128	<4	8
24	4714	8	<4	8	8	8	8	128	<4	64
25	7575	8	<4	8	16	8	8	256	16	512
26	1151	8	<4	16	8	8	16	256	<4	16
27	7314	8	<4	16	16	16	8	256	16	1024
28	3919	8	<4	16	16	32	16	256	<4	64
29	165	8	<4	32	16	16	32	1024	16	512
30	4706	16	<4	4	8	8	<4	1024	<4	8
31	7288	16	<4	<4	8	4	4	1024	<4	<4
32	8809	16	<4	4	16	8	16	512	8	128
33	7943	16	<4	<4	16	32	4	512	<4	<4
34	8639	16	<4	<4	32	16	8	1024	8	8
35	6240	16	<4	4	32	32	32	512	<4	<4
36	8345	16	<4	<4	32	64	32	2048	16	8
37	54/69	16	8	<4	8	8	8	1024	1024	<4
38	8419	16	16	<4	16	16	8	256	256	<4
39	6359	16	<4	16	32	32	32	512	64	1024
40	8055	32	<4	<4	8	4	<4	512	<4	<4
41	54/74	32	<4	<4	8	4	4	1024	<4	<4
42	5131	32	<4	<4	8	4	4	2048	<4	<4
43	7826	32	<4	<4	16	4	<4	512	<4	<4
44	2904	32	<4	<4	16	4	<4	4096	<4	<4
45	1155	32	<4	<4	16	8	4	2048	<4	<4
46	7977	32	<4	<4	16	8	16	512	<4	<4
47	7751	32	<4	<4	16	8	16	512	<4	<4
48	7476	32	<4	<4	16	16	16	512	<4	<4
49	7921	32	16	<4	8	8	8	1024	1024	<4
50	1348	64	<4	<4	16	8	8	1024	<4	64

Neutralizing antibody titers determined by the colorimetric method of Salk *et al.* (3) using a monkey kidney tissue culture system.

Comparison of the complement fixation test results using unheated antigen with the results of the neutralization tests in Table V shows that an occasional serum contained no demonstrable complement-fixing antibody to a virus type although neutralizing antibody to that type was present (for example, serum specimens 6, 9, 15, 22). On the other hand, with a single exception (serum 15), complement-fixing antibody was not demonstrable in the absence of the corresponding homologous neutralizing antibody. In marked contrast, comparison of the results of complement fixation tests using heated antigen with the results of the neutralization tests shows a very significant level of heterotypic fixation. Thus, 16 of the 50 sera gave fixation of 1:4 or higher with an antigen against

TABLE VI

Reactivity of Heated Poliomyelitis Virus Antigens. Fixation of Complement with Human Sera Containing No Demonstrable Neutralizing Antibody Corresponding to the Immunologic Type of Antigen Used

No. of sera tested	Results of complement fixation tests with heated antigens							
	Sera with no demonstrable neutralizing antibody* to	No. of sera giving fixation‡ with a *single* heterotypic antigen, *viz.*			Sera with no demonstrable neutralizing antibody* to	No. of sera giving fixation‡ with *two* heterotypic antigens, *viz.*		
		Type 1	Type 2	Type 3		Types 1 and 3	Types 2 and 3	Types 1 and 2
50	Type 1	0			Types 1 and 3	1 (2%)		
	Type 2		12 (24%)		Types 2 and 3		12 (24%)	
	Type 3			4 (8%)	Types 1 and 2			4 (8%)

* Neutralizing antibody titers of less than 1:4. (Colorimetric method of Salk *et al.* (3) using monkey kidney tissue culture system).

‡ Complement-fixing titers of 1:4 or more.

whose immunologic type there was no detectable neutralizing antibody in the serum and an additional 17 sera gave fixation (1:4 or higher) with two antigens against whose immunologic types there was no demonstrable neutralizing antibody.

The extent of the cross-reactions is summarized in Table VI by immunologic type of virus. It will be seen that of the sera which gave cross-reactions against a single virus type, no instances were encountered in which a serum with no demonstrable Type 1 neutralizing antibody gave fixation with a heated Type 1 antigen. However, 12 sera (24 per cent) containing no demonstrable Type 2 antibody gave fixation with a Type 2 antigen, and four sera (8 per cent) containing no Type 3 antibody gave fixation with a Type 3 antigen. With respect to fixation against multiple antigens, one serum containing neither Type 1 nor Type 3 antibody gave fixation with Type 1 and Type 3 antigens. Twelve sera (24 per cent) containing no Type 2 or Type 3 antibody gave fixation with Type

2 and Type 3 antigens, and four sera (8 per cent) containing no Type 1 or Type 2 antibody gave fixation with Type 1 and 2 antigens.

Summation of the left- and right-hand portions of Table VI, *i.e.* the number of sera which reacted heterotypically with one type and the number of sera which reacted heterotypically with two types, shows that in the series of 50 sera tested, 5, or 10 per cent, gave heterotypic fixation with Type 1 virus; 28, or 56 per cent, gave heterotypic fixation with Type 2 virus; and 17, or 34 per cent, fixed heterotypicaly with the Type 3 virus.

These 50 sera were tested on three different occasions with three different lots of Type 1 and Type 3 antigens and four different lots of Type 2 antigen, so that the differences between the results obtained with heated and unheated materials are not ascribable to some peculiarity in any one test or antigen. The loss of homotypic specificity of the heated antigens was apparently due to an alteration of the virus or its end-products rather than to a change in some component of the HeLa cell cultures, as the sera did not react with heated control antigens prepared from non-infected HeLa cell cultures.

Before attempting to interpret the significance of cross-reactions, ancillary data seemed desirable on the time of appearance of these heterotypic complement-fixing substances, whether rises in their level occurred, the titers encountered, and whether they tended to persist. To obtain information on these points, paired or multiple serum specimens from 20 patients with a clinical diagnosis of poliomyelitis were examined.

Of these 20 patients, one, an infant, apparently did not have poliomyelitis despite the clinical diagnosis, one patient had a Type 3 virus infection, and the remaining 18 had Type 1 virus infections. The results are presented in Table VII, which gives the complement-fixing antibody levels as determined with both heated and unheated antigen and the neutralizing antibody titers as determined by the colorimetric method using HeLa cells (4). The serum of patient 1, from whom no poliomyelitis virus was isolated, contained no demonstrable neutralizing antibody for the poliomyelitis viruses, nor was complement-fixing antibody detectable using either unheated or heated antigen.

Patient 2 had neutralizing antibodies only to the Type 3 virus, and this was reflected by the complement fixation test with the unheated antigen which showed no demonstrable complement-fixing antibody to Types 1 or 2 but a rise in titer to Type 3; the heated antigen, however, indicated a rise in Type 2 complement-fixing antibodies and a stationary titer to Type 3. (The titers obtained with the Type 1 heated antigen are within the experimental error (2-fold differences) of the method and are not regarded as significant.)

Of the 18 individuals with Type 1 infections, all showed 4-fold or greater rises in complement-fixing antibody titer with the unheated antigen except patients 17 and 20 in whom only 2-fold rises, not significant diagnostically, were encountered. It will also be seen that with the unheated antigen no heterotypic responses were encountered; in patient 5, the rise from less than 1:4 to 1:4 with the Type 3 antigen is not significant, nor is the 2-fold rise against the Type 3 virus in patient 7 significant. The appreciable amount of Type 3 complement-fixing antibody in patient 7 as revealed by the unheated antigen is compatible with the high neutralizing antibody present to this type. This situation is different from that encountered in this group of patients when heated antigen was used. Patients 4, 6,

TABLE VII

Loss of Homotypic Specificity of Poliomyelitis Virus Complement-fixing Antigens after Heat Treatment

Patient	Age	Sex	Days after onset	Clinical type of poliomyelitis	Virus isolated from stool	Poliomyelitis complement fixation titers						Poliomyelitis neutralizing antibody titer*		
						Unheated antigen			Heated antigen					
						Type 1	Type 2	Type 3	Type 1	Type 2	Type 3	Type 1	Type 2	Type 3
1. Sh.St.	10 mos.	F	7	Paralytic	None	<4	<4	<4	<4	<4	<4	<4	<4	<4
			36			<4	<4	<4	<4	<4	<4	<4	<4	<4
2. Li.Ro.	15 yrs.	F	7	Paralytic	Type 3	<4	<4	8	<4	4	16	<4	<4	128
			30			<4	<4	32	4	32	32	<4	<4	512
3. Ro.Mo.	31 yrs.	M	7	Paralytic	Type 1	<4	<4	<4	64	16	32	32	32	<8
			29			16	<4	<4	16	8	8	256	16	<4
4. Ca.Et.	8 yrs.	F	2	Paralytic	Type 1	<4	<4	<4	<4	8	8	<4	<4	<4
			17			16	<4	<4	16	64	64	256	<32	<32
5. Gr.Br.	31 yrs.	F	6	Non-par-	Type 1	<4	<4	<4	16	32	64	32	8	512
			25	alytic		16	<4	4	16	32	64	128	<4	512
6. Ka.McH.	8 yrs.	F	7	Contact	Type 1	<4	<4	<4	<4	4	4	<4	<4	<4
			21			8	<4	<4	16	32	32	64	<4	<4
			49			16	<4	<4	8	16	16	128	<4	<4
7. Be.Cu.	12 yrs.	F	5	Paralytic	Type 1	<4	<4	32	<4	8	16	<16	<16	128
			21			16	<4	64	16	16	32	128	64	128
			35			32	<4	64	16	16	32	512	128	128
8. Sa.Co.	2 yrs.	M	3	Paralytic	Type 1	<4	<4	<4	<4	<4	16	16	<4	<4
			27			32	<4	<4	32	<4	8	128	<4	<4
9. Ca.Cu.	4 yrs.	F	8	Paralytic	Type 1	<4	<4	<4	<4	<4	<4	<16	32	<16
			35			64	<4	<4	32	<4	8	512	16	<4
10. Ge.Wo.	2 yrs.	M	3	Paralytic	Not	<4	<4	<4	16	32	8	128	64	<4
			17		done	64	<4	<4	32	16	16	256	64	<4
11. Su.Gr.	1 yr.	F	10	Paralytic	Type 1	<4	<4	<4	<4	<4	<4	<4	<4	<4
			32			64	<4	<4	32	4	4	128	<4	<4
12. Ro.Sk.	6 yrs.	M	3	Paralytic	Type 1	4	<4	<4	64	32	32	32	<4	<4
			17			32	<4	<4	32	32	16	64	<4	<4
			31			32	<4	<4	32	32	32	128	<4	<4
13. Ma.Bo.	7 yrs.	F	8	Paralytic	Type 1	4	<4	<4	16	16	16	64	16	8
			22			32	<4	<4	32	16	16	128	<4	<4
			36			32	<4	<4	16	16	16	128	<8	<4
14. Wi.Bu.	9 yrs.	M	7	Paralytic	Type 1	4	<4	<4	64	32	32	8	<4	<4
			27			64	<4	<4	64	32	16	128	<4	<4
15. An.Go.	5 yrs.	F	6	Paralytic	Type 1	<8	<8	<8	32	16	16	64	32	<4
			17			8	<4	<4	16	8	8	256	64	8
			40			32	<4	<4	16	4	8	256	64	4

TABLE VII—*Concluded*

Patient	Age	Sex	Days after onset	Clinical type of poliomyelitis	Virus isolated from stool	Poliomyelitis complement fixation titers						Poliomyelitis neutralizing antibody titer*		
						Unheated antigen			Heated antigen					
						Type 1	Type 2	Type 3	Type 1	Type 2	Type 3	Type 1	Type 2	Type 3
16. Je.Wi.	29 yrs.	F	8	Paralytic	Type 1	<8	<8	<8	<8	16	64	8	<4	<4
			29			32	<8	<8	32	16	64	128	<4	<4
			36			16	<4	<4	16	8	32	128	<4	<4
17. Et.Sc.	34 yrs.	F	6	Paralytic	Type 1	8	<4	<4	16	32	16	32	8	<4
			27			16	<4	<4	16	16	8	128	8	<4
18. Do.El.	14 yrs.	F	10	Paralytic	Type 1	8	<4	<4	32	32	64	256	<4	<4
			36			32	<4	<4	32	16	32	512	<4	<4
19. Sh.v.Vl.	3 yrs.	F	18	Paralytic	Type 1	16	<4	<4	16	4	<4	64	<4	<4
			33			64	<4	<4	16	<4	<4	512	<4	<4
			49			64	<4	<4	16	<4	<4	1024	<4	<4
20. Mi.Ba.	2 yrs.	M	27	Paralytic	Type 1	32	<4	<4	16	4	32	128	<4	<4
			41			64	<4	<4	32	4	16	256	<4	<4

* Determined by the method of Lipton and Steigman (4), using a HeLa cell culture system.

and 9, for example, showed with the heated antigen a rise in complement-fixing antibody to a virus type to which they had no demonstrable neutralizing antibody. (The convalescent-phase serum of patient 4 was toxic to HeLa cells in dilutions up to 1:32, and it is assumed that no rise in neutralizing antibody to Type 2 and Type 3 viruses occurred. Even if this individual is removed from subsequent consideration in the computation of the total degree of heterotypic fixation encountered, the percentage of cross-reactions is not greatly altered.) In other individuals, antibody reacting with heated antigen was present early after the onset of the illness, the titer did not change, and the antibody was present at a fairly high level for 6 or 7 weeks at least.

The findings of the complement fixation tests on the 18 individuals with Type 1 infections may be summarized as follows: With the unheated antigen, two individuals had stationary complement-fixing antibody titers to the Type 1 virus, and the sera did not give cross-reactions with Type 2 and Type 3 antigens. One individual showed a rise in antibody titer to the Type 1 virus and possessed a stationary antibody titer to the Type 3 virus, against which he also possessed neutralizing antibodies. Fifteen individuals showed rises in titer to the Type 1 virus but no reactions to the Type 2 or Type 3 virus antigens. With the heated antigens, on the other hand, 10 individuals with no Type 2 or Type 3 neutralizing antibody gave fixation against these types, 3 of the 10 actually showing rises in titer to Type 2 and Type 3 viruses. Four additional individuals with no neutralizing antibody to the Type 3 virus gave fixation with the Type 3 antigen, 1 of these 4 showing a rise in titer to this type. The 10

cross-reactions with the Type 2 heated antigen represent an incidence of 56 per cent heterotypic reactions with the Type 2 virus, and the 14 individuals

TABLE VIII

Lack of Reactivity of Heated and Unheated Poliomyelitis Virus Complement-Fixing Antigens with Sera of Patients with Mumps Meningoencephalomyelitis

Patient	Days after onset	Mumps C-F titer*	Complement-fixing titers with						Neutralizing antibody titers‡		
			Unheated antigens			Heated antigens					
			Type 1	Type 2	Type 3	Type 1	Type 2	Type 3	Type 1	Type 2	Type 3
21. Bi.Ha.	6	<8	<4	<4	<4	<4	<4	<4	<4	<4	<4
	44	128	<4	<4	<4	<4	<4	<4	<4	<4	<4
22. Gl.Mo.	3	16	<4	<4	<4	<4	<4	<4	<4	<4	<4
23. Vi.Ma.	9	16	<4	<4	<4	<4	<4	<4	<4	<4	128
	30	64	<8	<8	<8	<8	<8	<8	<8	<8	128
24. Be.Ca.	6	16	<4	<4	<4	<4	<4	<4	128	32	128
	30	64	<4	<4	<4	<4	<4	<4	64	32	64
25. Mi.Co.	21	64	4	<4	<4	<4	<4	<4	512	<4	<4
26. Da.Be.	10	16	32	<4	<4	8	<4	<4	128	128	8
	28	128	32	<4	<4	4	<4	<4	128	32	<4
27. Ja.Mc.	41	256	<8	8	<8	<8	16	<8	<16	512	<4
28. Do.Al.	3	<8	<4	64	<4	<4	64	<4	128	>2048	128
	23	16	<4	64	<4	<4	64	<4	128	>2048	128
29. Sa.Su.	16	64	<4	<4	<4	4	4	<4	128	256	32
	29	64	<4	<4	<4	4	8	<4	128	256	64
30. Je.Ja.	15	32	<4	<4	<4	8	4	8	<4	32	8
	76	256	<8	<8	<8	<8	<8	<8	<4	4	<4

* Antibody titer to the S (soluble) antigen of the mumps virus.

‡ Titers determined by the method of Salk *et al.* (3) using monkey kidney tissue culture system.

giving cross-reactions with the Type 3 heated antigen represent an incidence of 78 per cent heterotypic fixation with this type.

These findings indicate that the heterotypically reacting substances appear very early during the course of the infection and are generaly at their maximal level at the time of onset of the disease, although in some instances they appear with the onset of the illness, and increase in titer, as does the homotypic anti-

body. Also, they do not disappear promptly but persist for at least some weeks and probably longer. The remarkably high incidence of heterotypic reactions in these patients together with the 50 individuals described above suggests that the Type 1 virus is immunologically related to Types 2 and 3 and that heat treatment of the homotypically specific native antigen uncovers a reactive group shared by all three virus types. The poliomyelitis infections we have seen have been due preponderantly to Type 1 virus; we have encountered only a few Type 3 virus infections and fewer Type 2 infections, and studies on individuals infected with these two viruses should help to elucidate further the antigenic relationships suspected by others, and supported by these findings, to exist be-between the three immunologic types of poliomyelitis virus.

Specificity for Poliomyelitis Virus of the Reactions with Heated Antigens.—The sera which reacted heterotypically with the heated poliomyelitis antigens gave no fixation with the heated control preparations. This suggested that the heterotypic reactions were specific in the sense that fixation of complement occurred only in the presence of some reactive moiety possessed by the poliomyelitis group of viruses. Evidence for such group specificity was afforded by the results of complement fixation tests with sera from patients in whom the clinical diagnosis was non-paralytic poliomyelitis, but who were subsequently shown to have mumps virus infections—no virus was recovered from the stools, and there was a rise in the antibody titer to the S (soluble) antigen of the mumps virus.

Seventeen sera from 10 such patients with mumps meningoencephalomyelitis were tested with heated and unheated poliomyelitis complement-fixing antigens, and the results are given in Table VIII. The sera of 4 patients (Nos. 21 through 24) gave no reactions to any virus type with either heated or unheated antigens. 2 of these individuals possessed no demonstrable neutralizing antibody to any of the three virus types, 1 had neutralizing antibody to Type 3 virus only, and 1 had neutralizing antibody to all three types. The serum of patient 25 gave fixation (minimal) only with the unheated Type 1 antigen, and the only demonstrable neutralizing antibody in this serum was to the Type 1 virus.

Patient 26 gave fixation with both unheated and heated Type 1 antigen, but not with antigens to the other types; patients 27 and 28 similarly gave fixation with both unheated and heated Type 2 antigens, but not with those of other virus types. In each instance neutralizing antibody corresponding to the immunologic type of the antigen which gave fixation was present.[5]

[5] The exact diagnosis in patient 28, however, is uncertain. The high complement-fixing and very high neutralizing antibody titers to the Type 2 virus point to this agent as the cause of the current illness. If this interpretation be made, then the rise, at a low level, of S antibody to the mumps virus must be regarded as evidence of a dual infection or as a non-specific reaction; in the latter case, the diagnostic significance of mumps S antibody is called into question. On the other hand, the stationary poliomyelitis antibody titers may reflect a recent infection with Type 2 virus and the rising mumps antibody, current infection with this agent. This latter interpretation was made here and was based in part on the presence of a high Type 2 complement-fixing antibody titer at 3 days after the onset of illness; poliomyelitis complement-fixing antibody is generally slow in developing, and titers of this magnitude are not commonly encountered so soon after onset.

Unheated antigens gave no fixation with the sera of patient 29, but borderline reactions occurred with heated Type 1 and Type 2 antigens. Since neutralizing antibody to all three virus types was present in the sera, and since cross-fixation can, as shown elsewhere above, occur between types in these circumstances, the results are not unexpected. The findings with the sera of patient 30, however, are not readily explicable and should, in the interests

TABLE IX

Attempts to Separate, by High Speed Centrifugation, Fraction Responsible for Cross-Reactivity of Heated Poliomyelitis Complement-Fixing Antigens

Antigen		Fractions tested for antigenicity before and after centrifugation at 104,000 *G* for 3 hrs.	Antigenic titer of fraction with homologous monkey immune serum
Virus type	Treatment		
Type 1 (Mahoney	None*	1. Tissue culture fluid	1:8
		2. Supernatant fluid	0
		3. Sediment (resuspended pellet)	1:4
	Heated‡	1. Tissue culture fluid	1:6
		2. Supernatant fluid	0
		3. Sediment (resuspended pellet)	1:3
Type 2 (Statler)	None	1. Tissue culture fluid	1:8
		2. Supernatant fluid	0
		3. Sediment (resuspended pellet)	1:6
	Heated	1. Tissue culture fluid	1:3
		2. Supernatant fluid	0
		3. Sediment (resuspended pellet)	1:2
Type 3 (McMullen)	None	1. Tissue culture fluid	1:4
		2. Supernatant fluid	0
		3. Sediment (resuspended pelet)	1:3
			1:3
	Heated	1. Tissue culture fluid	1:4
		2. Supernatant fluid	0
		3. Sediment (resuspended pellet)	1:3

* Infectious, untreated material; *i.e.*, native antigen.

‡ Infectious culture fluid heated at 56°C. for 30 minutes.

of objectivity and in the light of the meager current knowledge, be regarded as constituting non-specific reactions.

In so far as this small group of individuals is concerned, the observations on the whole indicate that the reactivity of heated poliomyelitis antigens is essentially specific for the poliomyelitis group of viruses. When complement-fixing antibodies were demonstrable with the use of native unheated antigen, neutralizing antibody of the corresponding immunologic type was also found present, and the heated antigen (with one exception) also gave similar reactions.

In two instances, the heated antigen gave minimal degrees of fixation, while the unheated antigen did not give any fixation; one of these reactions seems attributable to the neutralizing antibodies present in the serum, the other is regarded as non-specific.

Factor Responsible for Heterotypic Fixation of Heated Antigens.—

Black and Melnick (12) observed that formalinization of their live virus antigens resulted in a variable loss of homotypic titer and an increase in cross-reactions between the poliomyelitis virus types. Also, while most of the complement-fixing antigen in the untreated preparations could be sedimented by high speed centrifugation, formalin treatment released a "soluble antigen" which remained in the supernatant fluid.

Heated and unheated antigens (infected HeLa cell culture fluids) were centrifuged at 104,000 gravity for 3 hours. The supernatant fluids were completely removed and the sediments were resuspended to the original volume with Kolmer saline solution. The original starting materials, the supernatant fluids, and the resuspended sediments were titrated for complement-fixing antigenicity with homologous monkey immune sera. The complement-fixing titers of the various preparations are given in Table IX.

Under the conditions of these experiments, no evidence of a soluble antigen such as that encountered by Black and Melnick (12) was found. The complement-fixing activity of the centrifuged preparations was confined to the sediments; the supernatant fluids exhibited no demonstrable activity. It has yet to be determined, however, whether the heat treatment employed was not sufficiently stringent to release the factors uncovered by formalinization, despite the fact that the treatment was adequate to induce a heterotypic reactivity of the antigens.

DISCUSSION

If the complement fixation test for poliomyelitis is to be utilized by clinical and public health laboratories unfamiliar with the handling of viruses, elimination of the potential infection hazard is desirable. It was considered that this might be achieved by heating the virus-containing tissue culture fluids, which constitute the antigen, for 30 minutes at 56°C. It was found, however, that such treatment, while it does not completely destroy the complement-fixing capacity of the antigen, does adversely affect its potency and sensitivity. The loss in antigenic potency encountered was of the magnitude of 25 to 30 per cent and losses as high as 50 per cent were encountered. Such losses of activity militate against the use of heat as a means of abolishing infectivity, and are not in accord with reports that heat does not significantly reduce antigenicity.

Aside from the reduction in complement-fixing capacity and in sensitivity, heat treatment did not seem otherwise to affect the antigens in so far as tests with monkey immune sera were concerned. The heated antigens gave virtually as sharply homotypic fixation as did the unheated preparations with both homotypic and heterotypic monkey immune sera. Others (5, 6, 12) have reported similar experiences. It would thus appear that this absence of cross-reactivity of heated antigens when tested with monkey immune sera, either in

experimental work or for purposes of titrating the preparations for antigenic unitage, is the basis for reported impressions that homotypic specificity is unaffected by heat treatment.

However, with human sera there is a marked difference in homotypic specificity between heated and unheated antigens. Our unheated antigens tended rather uniformly to give homotypic fixation, whereas the heated antigens gave a very high proportion of cross-reactions. In a series of 50 single serum specimens, the incidence of heterotypic reactions was 10 per cent to Type 1 virus, 56 per cent to Type 2 virus, and 34 per cent to Type 3 virus. When paired or multiple serum specimens from patients with a clinical diagnosis of poliomyelitis were tested, no cross-reactions were encountered with the Type 1 virus, but the incidence of heterotypic fixations to the Type 2 virus was 56 per cent and to the Type 3 virus, 78 per cent. The loss of homotypic specificity, or conversely, the broad reactivity engendered by heat treatment of the poliomyelitis complement-fixing antigens, explains the heterotypic fixation reported by a number of investigators, who use heat-treated antigens.

Svedmyr (10), for example, reported that the complement fixation test was positive in 90 per cent of the cases of poliomyelitis tested, but the incidence of homotypic reactions was only 10 per cent. Black (9) has observed that 90 per cent of poliomyelitis patients possess complement-fixing antibodies against two or three types of the virus, and that most individuals who give heterotypic fixation have neutralizing antibody to more than one type, but not necessarily to the same type as their complement-fixing antibodies. His experience (9) agrees with that of others, and is supported by our findings with heated antigens, that complement-fixing antibody may be present very early in the disease and show no subsequent rise in titer, and that changes in titer between multiple sera may occur in almost any combination. Thus, "the homotypic titer may rise as the heterotypic falls; the titer against more than one type may rise simultaneously; or one or another type may be approximately the same in acute and convalescent specimens" (9). Black also summarizes the experience of others when he points out that while the type of complement-fixing antibody showing the largest rise in titer frequently corresponds with the type of the virus isolated from the stool, there are exceptions; hence, such serologic results cannot be depended upon as identifying the type of the infecting virus.

On the basis of the experience recorded in this paper, we consider it necessary, pending development of a method that will inactivate the virus without broadening the reactivity of the antigen, to use fully infectious antigens if heterotypic reactions are to be avoided and if identification of the infecting virus type is desired. Heated antigens may, perhaps, have some virtue for early diagnosis (2, 11) since antibody to any virus type or types may be detectable at, or shortly after, onset of the disease. However, the question arises whether, in any particular patient, the mere presence of heterotypic antibody, in either low titer or at a high stationary level, is diagnostically significant *per se*, *i.e.*, in the absence of supportive clinical, epidemiologic, or laboratory evidence;

poliomyelitis complement-fixing antibodies may persist for some months after infection (unpublished data), and thus may be present, and their significance misinterpreted, in patients suffering from diseases other than poliomyelitis (*cf.* Table VIII and references 1 and 16). In epidemiologic studies, on the other hand, the relatively short persistence of complement-fixing antibody suggests that its presence may be regarded as evidence of recent, not necessarily current, infection. Since, in such studies, knowledge of the specific infecting type is a desideratum, homotypically specific antigens are preferable to heterotypically reactive preparations.

Sabin (14) has observed that patients with a Type 1 poliomyelitis virus infection may develop a minimal and transitory heterotypic neutralizing antibody response to the Type 2 virus; this association has been interpreted to indicate an antigenic relationship between at least some strains of the Type 1 and Type 2 poliomyelitis viruses. It has also been suggested (6, 15) that heterotypic fixation of complement is based on the existence of an antigenic relationship between all three poliomyelitis virus types. The work reported here adds additional evidence for the existence of an antigenic overlapping between types. Also, this sharing of a common antigen(s) may perhaps explain Melnick's (17) finding of complement-fixing antibodies in the sera of normal children without demonstrable neutralizing antibodies, namely, that the complement-fixing antibodies represent a heterotypic response induced by a Type 4 poliomyelitis virus (17). It would be of interest to ascertain whether any agents of the "orphan" group can give rise to such heterotypic responses.

Since the unheated antigens reacted homotypically but became highly cross-reactive after heating, the treatment must have brought about a broadening of the normally type-specific reactive component, or exposed a group-reactive moiety otherwise not in evidence. Other observations also indicate that physical methods can bring about a change from type-specific reactivity to group-specific reactivity in the poliomyelitis viruses. LeBouvier (2), for example, observed that ultraviolet irradiation, like heat, bestows a cross-reactivity on previously type-specific complement-fixing antigens. We (unpublished data) and others (7) have observed that whereas monkeys inoculated with active poliomyelitis virus respond with the production of homotypic complement-fixing antibodies, animals inoculated with monovalent formalinized virus preparations tend to produce heterotypic complement-fixing antibody.

What the nature of the group-reactive substance may be is still unclear. Black and Melnick (12) found that while their active virus preparations were almost completely type-specific, formalinization made the antigens cross-reactive. Moreover, most of the complement-fixing activity in the live virus preparations could be sedimented by high-speed centrifugation, whereas in the formalinized preparations, considerable complement-fixing activity remained in the supernatant fluid, suggesting the presence of a "soluble" antigen. Similar attempts by us at centrifugal separation of a reactive fraction from heated preparations yielded no indication that a soluble antigen was involved. It is possible that

formalinization brings about changes not elicited by heat treatment as employed by us, although such treatment was adequate to induce a high degree of cross-reactivity.

Finally, the cross-reactivity conferred on poliomyelitis complement-fixing antigens by heating appears to be essentially specific for the poliomyelitis group of viruses. That it is not a non-specific reactivity is suggested from preliminary experiments in which the heterotypically reactive heated antigens did not fix complement in the presence of sera from patients with mumps meningoencephalomyelitis unless these sera also contained neutralizing antibody to one or another of the three immunologic types of poliomyelitis virus.

SUMMARY

Fluid obtained from HeLa cell cultures infected with poliomyelitis viruses served as a complement-fixing antigen. When used in the native state, *i.e.* untreated in any way, the fluids acted as homotypically specific antigens. When heated, however, the antigenicity was broadened and a high degree of heterotypic reactivity was encountered.

Data are presented indicating that the observed group reactivity was apparently based on common antigens shared by the three immunologic types of poliomyelitis virus. This reactivity appeared to be specific for the poliomyelitis viruses. No evidence was obtained in preliminary experiments that heating of the antigens releases a "soluble" antigen responsible for the group reactivity.

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