Summary. Fractions of human plasma prepared by methods 6 and 9 of Cohn and his associates have been assayed for ACTH activity by the procedure of Nelson and Hume. ACTH activity was found in Fraction II + III and occasionally in Fraction IV-1 and Fraction IV-4. Fractionation of human plasma to which ACTH had been added *in vivo* and *in vitro* gave similar results.

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Rapid Slide Precipitin Microreaction of Poliomyelitis Antigens and Antisera in Agar.* (23649)

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The precipitation of viral suspensions by specific antisera has been applied to plant viruses, and recently also to animal viruses. Belyavin(1) described the precipitation of influenza virus in aqueous suspension by means of specific antisera. Wilson Smith *et al.*(2) reported a flocculation reaction with polioviruses. Since their original method required large amounts of antigen, the same authors developed a micromethod of immuno-precipitation of polioviruses in aqueous medium in capillary tubes(3). Le Bouvier(4), as well as the present authors(5) independently described the use of the Ouchterlony method of double diffusion in an agar gel(6) for the study of the reaction between polioviruses and specific antisera. Since the usual technic in Petri dishes requires large quantities of antigen, a micromethod, described elsewhere(7) for the study of venoms and other antigens, was applied to the polioviruses.

Materials and methods. Routine microscopic slides, cleaned in alcohol, are used. Each slide is covered with 3 ml of a 1% hot agar solution. After solidification, 2 mm holes are punched in the agar with a special instrument. A fixed pattern of 6 holes disposed around a seventh, as shown in Fig. 1, proved to be very useful. The volume of each hole is approximately 4 cu. mm. *Agar solution.* A 2% solution of commercial, pure agar in distilled water is clarified by precipitation with egg albumin at 120°C. For use, this agar solution is melted and diluted to 1% in a M/7.5 phosphate buffer solution (Sørensen) and while very hot poured on the slides. The final pH should be 7.2. This concentration of agar was chosen in accord with Polson's data(8), because it is the minimal amount of agar giving a sufficiently solid sheet. *Antigens.* Cultures of KB cells in large flasks are massively infected with the respective type strains in use here (type 1 = strain No. 6,186 from Dr. Goffe; type 2 = strain MEF_1; type 3 = strain Saukett). In

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a medium consisting of 0.5% lactalbumin hydrolysate in Earle's solution, supplemented with 10% calf serum, the degeneration of the cells is almost complete in 24 hours. The flasks are then frozen and thawed, and the contents are clarified by 2 centrifugations at 3,200 rpm for 20 minutes. The viruses are then concentrated by centrifugation on the Spinco preparative machine at 30,000 rpm for 90 minutes using lusteroid tubes in the 40 rotor. The supernatant fluid is discarded, the pellets are suspended in PBS buffer(9) and left at 4°C overnight. Repeated pipetting then yields a uniform suspension. The tubes are rinsed with PBS and the pooled suspensions are adjusted to give a 50-fold concentration. This antigenic suspension is opalescent and contains no gross particles. Titration of the original suspension should give titers of at least 10^7 $TCID_{50}$ per ml. The antigens were kept at 4°C. *Sera.* As reference sera, we used the 3 type specific sera from hyperimmunized monkeys prepared by the National Foundation for Infantile Paralysis, and kindly distributed by this organization, to which we are indebted for their generous assistance. The study included also sera from patients with a clinical history of poliomyelitis, from healthy and vaccinated children and from animals (guinea pigs and rabbits) immunized with increasing volumes of Salk's vaccine in 3 courses of biweekly injections. Human gamma globulins were also used. *Procedure for reaction.* The holes in the agar are filled with the reagents by means of a doubly drawn, fine-tipped Pasteur pipette. The fundamental reaction unit consists of 2 holes containing respectively antiserum and antigen. The hexagonal array gives a constant distance between all the holes in the agar and thus gives several possibilities of reaction units. For the purpose of economy, it is advisable to use the central hole as one member of several reaction units, the other members being in each of the peripheral holes (Fig. 8). The slides are then put in a tightly closed glass chamber, containing an atmosphere that is saturated with water vapor by means of moistened blotting paper at room temperature (20°C). Tests were also made at 37°C with a slight gain in the speed of the reaction. After 16 to 24 hours the precipitation lines which appear in the agar separating the two holes containing the specific reagents may be observed by macroscopic dark field illumination. These lines show a tendency to regress when the diffusion time is prolonged. *Drying and staining.* The slides are arranged on a glass plate and covered with a sheet of moist blotting paper, which should adhere closely to the agar surface. Care must be taken not to introduce or to leave air bubbles in the holes, because they may cause breaks in the agar sheet during desiccation. The preparations are thus kept overnight in an incubator at 37°C. They can be kept dry as long as one wants. For staining, one moistens the blotting paper with water until it can be removed. The surface of the slides is cleaned with moist cotton wool or under gentle running water. This procedure allows the agar sheet to absorb the amount of water that is needed for staining. Experience has to be gained in achieving the right degree of humidity. Slides are introduced for 2 to 3 minutes in the staining mixture, then for several seconds in a series of 4 to 5 washing baths of similar composition (the baths contain one of the solvent mixtures A or B according to the dyes used). They are kept a little longer in the last bath with manual agitation. The time for the total differentiation is only a few minutes. This procedure should produce almost complete discoloration of the agar sheet and keep the lines stained. Precise time relationships cannot be furnished, because the amount of dye in the washing baths influences the speed of washing. The last bath should be renewed frequently. *Staining and solvent mixtures.* Several dyes can be used. The following substances were found particularly suitable: *Amidoschwartz 10 B* (Bayer), 0.1% in the solvent mixture A. *Azocarmin B* (National Aniline Division), 0.5% in solvent mixture A. *Bromophenol Blue,* 1% in solvent B (after staining and differentiation, the color of the preparation is developed in ammonia vapor). *Solvent Mixture A*: Methanol 98%, 50 parts; Distilled water, 40 parts; Acetic acid, 10 parts. *Solvent B*: Ethanol 70%.

Results. a) Reference sera. When undiluted reference monotypic sera are used in

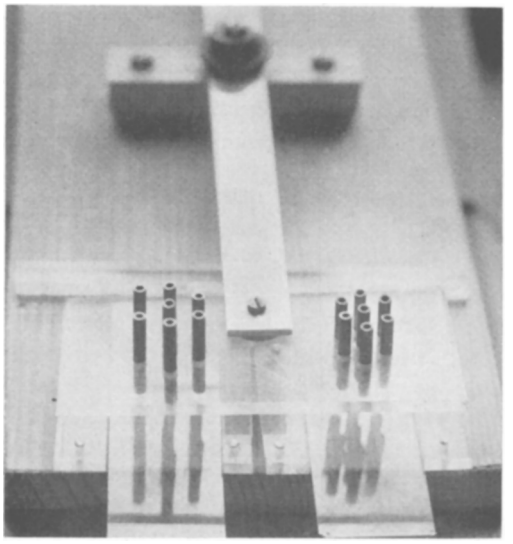


FIG. 1. Equipment used for punching holes in the agar.

this system, one observes one or two precipitation lines only with the antigen of the corresponding type. Two lines differing in intensity were observed in the type 1 (Fig. 3) and in the type 2 systems (Fig. 4), but only one in the type 3 system of precipitation (Fig. 5). The use of diluted sera shows that the precipitation lines occur at the ⅛ dilution, but are less apparent at the 1/16 dilution (Fig. 8).

b) *Gamma globulins of human origin.* With pooled gamma globulin of American or Swiss origin, precipitation lines were observed with all three types of antigen (Fig. 6). If the same solution of gamma globulin diffuses against the 3 separate types of antigen, placed in adjacent holes around the globulin, the precipitation lines cross each other. On the other hand, when several adjacent holes are filled with the same antigen, the gamma globulin gives rise to a continuous line of precipitation (Fig. 7). The main antigen or antigens of each type of poliovirus is clearly different as seen by this method.

c) *Animal sera.* The sera of rabbits and guinea pigs vaccinated with the Salk vaccine give rise to precipitation lines against the 3 monotypic antigens. No reactions were obtained with control antigens prepared from extracts of uninfected KB or monkey kidney

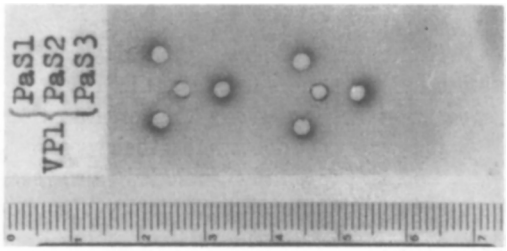


FIG. 2. Slide showing micro-reactions after staining.

TABLE I. Neutralizing and Precipitating Antibodies for Type 1 Poliovirus in Paired Sera.

Type of individual	No.	Before vaccine NA 4*	NA 40	PA Und.	PA 2	PA 4	PA 8	After vaccine† NA 4	NA 40	PA Und.	PA 2	PA 4	PA 8
Healthy children	1	0	0	0	0	0	0	0	0	0	0	0	0
inoculated with	2	0	0	0	0	0	0	0	0	++	+	+	0
3 doses of killed	3	0	0	+	+	0	0	0	0	+	+	+	0
poliovirus vac-	4	0	0	+	+	+	0	+	+	++	++	++	+
cine	5	0	0	++	++	+	0	+	+	++	++	++	+
	6	0	0	0	0	0	0	+	+	0	0	0	0
	7	0	0	0	0	0	0	+	+	+	0	0	0
	8	+	+	±	0	0	0	+	+	++	++	+	0
	9	+	+	+	0	0	0	+	+	++	+	0	0
	10	+	+	++	+	0	0	+	+	++	++	+	0
	11	+	+	+	+			+	+	++	++	++	
	12	+	+	++	++	+	0	+	+	++	++	++	+
	Serum												
"Non-polio" lymph-	Acute	+	0	0	0	0	0						
ocytic meningitis	Conval.	+	0	0	0	0	0						
Type 1 paralytic	Acute	+	+	++	+	+	0						
poliomyelitis	Conval.	+	+	++	++	++	+						

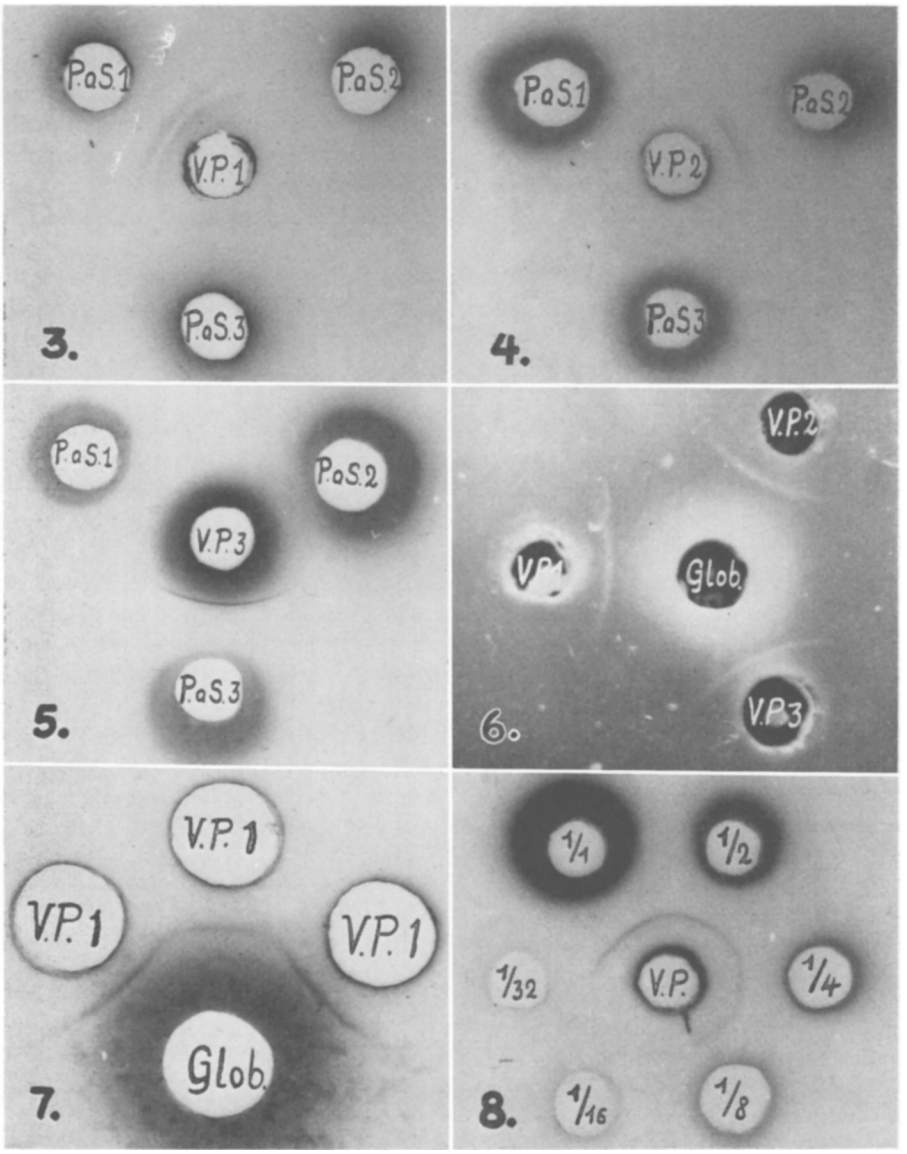
NA = neutralizing antibody; PA = precipitating antibody. In precipitation tests, + = definite reaction and ++ = heavy precipitation.

* Dilution of serum tested. † 4 wk after 3rd dose.

cells. Sera from non-immunized rabbits and guinea pigs gave no reaction with poliovirus antigens.

d) *Human sera.* Twelve pairs of sera of children, taken before and after vaccination with 3 injections of a killed poliovirus vaccine were submitted to the precipitation test. One pair of sera (early and convalescent) from a child suffering from lymphocytic meningitis of unknown, but surely not poliomyelitic origin, and one pair of sera (10- and 20-day) of a paralytic case of type 1 poliomyelitis were also tested. Results are shown in Table I.

Among the 7 children, who before vaccination had no neutralizing (N) antibody in the low dilution of serum tested, 3 (Nos. 3, 4 and 5) gave positive tests for precipitating (P) antibody. Control tests with antigens from



FIGS. 3 to 8. Enlarged photographs of different stained precipitin micro-reactions, except Fig. 6 observed unstained in darkfield. V.P. 1 = poliovirus type 1; V.P. 2 = poliovirus type 2; V.P. 3 = poliovirus type 3; P.aS. 1 = poliomyelitis antiserum (monkey) type 1; P.aS. 2 = poliomyelitis antiserum (monkey) type 2; P.aS. 3 = poliomyelitis antiserum (monkey) type 3; Glob. = human gamma globulin.

uninfected KB cells were negative. Whether this is due to the presence of group-specific P antibodies in human sera for group-specific poliovirus or other viral antigens or whether in some individuals the type-specific P antibody may be a more sensitive indicator of previous infection with a specific type of poliovirus than the cytopathogenic test for N antibody used here, is not clear from the available data. The failure of child No. 3 of this latter group, who exhibited only P antibodies prior to vaccination, to develop any N antibodies after 3 doses of vaccine is not compatible with the assumption that the prevaccination P antibodies in this child were due to a previous infection with Type 1 poliovirus or for that matter with any poliovirus. Of the 7 children who exhibited no Type 1 N antibodies prior to vaccination, 3 failed to develop such antibodies after 3 doses of this particular killed virus vaccine, while one (No. 2) did develop P antibodies. Of the two children (Nos. 6 and 7) who exhibited neither N nor P antibodies prior to vaccination both developed N antibodies but only one exhibited a trace of P antibody after vaccination. There was thus no indication that the precipitin test was just another way of measuring N antibody. Among the 5 children who had Type 1 N antibody prior to vaccination all exhibited some P antibody before the vaccine and an increase in intensity of the precipitin reaction as well as a 2-fold increase in titer after the vaccine. A similar increase in the activity of the P antibody was observed in the sera from the patient with poliomyelitis, while no P antibody appeared in the sera of the one patient with lymphocytic meningitis of non-poliomyelitic etiology. Many other single serum specimens from patients with poliomyelitis were also tested and all reacted strongly in the precipitation test.

Stability of the antigen. The live concentrated virus suspensions were stored at 4°C for more than 2 months without loss of precipitating activity. In view of the potential danger of infection with these concentrated antigens, preliminary tests were carried out with formalinized antigens. Although the formalinized antigens appeared to be as potent as the live virus antigens, they lost the capacity to yield the double precipitation lines observed with types 1 and 2 antisera, and the single line that occurred was more diffuse than with the live virus antigens.

Discussion. The type specificity of the precipitation lines produced in agar when poliovirus antigens were allowed to react with antibody was clearly evident when monkey antisera were used. The presence of Type 1 P antibody in the sera of 3 of 7 children who had no type 1 N antibody in the low dilution of serum tested, indicates that the question of group-specific P antigens and antibodies needs to be investigated. It is well known that group-specific, complement-fixing antibodies are readily demonstrable in human sera but not in monkey antisera containing high titers of N antibody. It is already evident that the precipitation test is not merely another way of measuring N antibody but the relationship between the P antibodies and the type-specific and group-specific complement-fixing antibodies still remains to be determined.

Summary. 1. A simple and rapid technic is described for demonstrating a precipitin reaction in agar between minute amounts of poliovirus antigens and antisera. The preparations can be dried and stained and preserved indefinitely. 2. With monkey antisera the reaction was type-specific, while human sera were encountered which exhibited the precipitating antibody in the absence of readily demonstrable neutralizing antibody for the same type of poliovirus. 3. Sera from patients with poliomyelitis and human gamma globulin regularly exhibited the precipitin reaction.

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Metabolism of Human Amnion Cell Cultures Infected with Poliomyelitis Virus. I. Glucose Metabolism During Virus Synthesis.¶ (23650)

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Human amnion cell cultures have been shown to be highly susceptible to infection with various polioviruses(1,2). Thus, a convenient system is at hand for studying metabolic changes in host cells during infection with the virus. In this work glucose uptake of infected and control cell cultures was measured, and with the aid of various metabolic inhibitors reactions important for virus reproduction were indicated.

Materials and methods. Preparation of amnion cell cultures. The technic deviates in a number of details from procedure described by Zitcer *et al.*(1), and modifications by Takemoto and Lerner(3), Weinstein *et al.* (4) and Lahelle(5). Human placentas† collected in sterile towels were brought to laboratory within a few hours after delivery. The amnion membrane was stripped off, washed several times with phosphate buffered saline (PBS) and blood clots were removed. The membrane was then cut into 3-4 pieces, put in Erlenmeyer flasks, and covered with pre-warmed Hanks' salt solution containing 0.25% trypsin (N.B. Co. 1/300), pH-7.6. After 1 hour, trypsin was poured off and replaced by fresh pre-warmed 0.1% trypsin solution (pH-7.6) and the amnion left overnight at room temperature. Flasks were always kept tightly stoppered. The next morning fresh trypsin solution was added and the flasks vigorously shaken by hand. This procedure was repeated 3-4 times, employing fresh (0.1%) trypsin solutions each time, till most cells were set free. The combined fluids were centrifuged at 800 rpm 8 minutes, and the sediment washed twice with sterile PBS. Total count of cells was determined in hemocytometer after staining with crystal violet solution(6), while percentage of non-viable cells was estimated from another sample stained with trypan blue(7). Suspensions showing more than 10% cells stained with trypan blue were discarded. Finally, cells were resuspended to contain 500,000/ml in a medium consisting of 4 parts of 0.5% lactalbumin hydrolysate in Hanks' solution and 1 part of calf serum. The pH of medium was adjusted to 7.4. As seed 0.5 ml of this suspension was used for tubes, and 13 ml and 50 ml for milk bottles and Roux flasks, respectively. All containers were stoppered with rubber and incubated at 37°C; tubes were kept in slightly slanted position. After 48 hours the medium was replaced by Hanks' solution containing 0.5% lactalbumin hydrolysate to which 2% calf serum and 0.5% chick embryo extract were added; 1 ml of this medium was dispensed into tubes. A confluent sheet of cells was obtained within 3 to 5 days. The maintenance medium for these cultures was composed as follows: 0.5% lactalbumin hydrolysate in Earle's salt solution and 2% of calf serum. All solutions and media contained 100 units of penicillin and 100 μg of streptomycin/ml. Mycostatin (30 units/ml) was added to membranes during

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