network of the fibrin s clot. When calcium is present, fibrin-stabilizing factor (present in most fibrinogen preparations) gives rise to fibrin i by cross-linking these fibrin strands and thereby making them resistant to the action of solubilizing agents. The data suggest that there may be additional, and possibly physiological, substances whose fibrin s solubilizing ability has not yet been reported. Therefore, to avoid possible misinterpretation of fibrinolytic activity data obtained with the fibrin plate method it would appear essential that a) only fibrin i preparations be used in such tests, and b) the ionic concentration of the fibrinogen solution used be carefully standardized and controlled.

Summary. Solubilization of bovine fibrin s preparations by phosphatidyl inositol and phosphatidyl serine has been observed whereas phosphatidyl ethanolamine and lecithin did not have this ability. The solubilizing effect did not occur with preparations of bovine fibrin i. The extent of solubilization progressively diminished with increasing ionic concentration of the system, but was not significantly influenced by the type of buffer. The importance of using only fibrin i preparations in the fibrin plate assay for fibrinolytic or activator activities and the need for careful control of the ionic strength of the assay system are stressed.

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Isolation and Properties of a Human Enterovirus, Non-Pathogenic for Monkey Kidney Cell Cultures and Suckling Mice.* (28158)

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During studies on the association of enteroviruses with human diarrheal disease a virus was isolated on April 14, 1959, in human amnion (FL line) cell cultures from the stool specimen of a 9-month-old male child. The virus proved to be non-pathogenic for monkey kidney cell cultures and for suckling mice. It could not be identified as any of the recognized human enteroviruses. The original specimen (in the form of a rectal swab) was sent to the laboratory by accident; the patient was actually admitted to the Children's Hospital, University of Pittsburgh Health Center, because of an acute upper respiratory illness of undetermined etiology. Due to absence of diarrheal disease acute and convalescent serum specimens were not obtained.

Materials and methods. Source of viruses and immune sera. Poliomyelitis Type 1-3, Coxsackie A 7, 9, B 1-6, ECHO 1-27 viruses were kindly supplied by Dr. D. S. Yohn; ECHO 28 virus and immune serum by Dr. W. J. Mogabgab; E 59 (JV 10) virus and immune serum by Dr. W. C. Branche; Coxsackie A 21 (Coe) virus and immune serum by Dr. E. H. Lennette; the Pett virus(20) by Dr. J. A. Kasel, and Coxsackie A 2,

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3, 8, 10, 11, 12, 13, 14, 15, 16, 17, viruses adapted to human amnion primary culture cells by Dr. H. A. Wenner. Some of the non-prototype but enterovirus-like viruses were also tested for identification. HSO virus and immune serum(1) were kindly supplied by Dr. M. Matumoto, the Frater virus(2) by Dr. P. Kamitsuka, the Giles virus and immune serum by Dr. M. Cooney(3), the PR 10, 17, 20, 22, 28 viruses and immune sera (4,5) by Dr. W. C. Branche. Several other viruses isolated in this laboratory were also tested(6,7,8). Immune sera, prepared in rabbits against Poliomyelitis Type 1-3, Coxsackie B 1-5, ECHO 1-6, 8-10, 12-15 were purchased from the Microbiological Associates, Inc., Bethesda, Md. Coxsackie A 1-19, ECHO 7, 11, 16-25 immune sera prepared in monkeys were furnished by Dr. J. T. Syverton; Coxsackie B6, ECHO 26, 27 monkey immune sera were donated by Dr. D. S. Yohn; ECHO 28 rabbit immune serum was given by Dr. W. J. Mogabgab. Immune sera against Coxsackie A 20, 20/a, A 21 (Coe), and Pett were also prepared in monkeys in this laboratory.

Tissue cultures. Trypsin dispersed monkey kidney tissue cultures were prepared by a modification of Youngner's method(11). Human amnion, FL line cells(12), were maintained in 32 oz bottles by weekly passage. Hanks' Balanced Salt Solution with 0.5% lactalbumin hydrolysate and 10% bovine serum was used as growth medium. Tissue culture tubes were prepared by inoculating 25,000 cells per tube in 1 ml of medium. The tubes were ready for use on the 4th-5th day. Eagle's medium (bovine amniotic fluid plus 2% bovine serum) was used as maintenance medium. The same medium without serum with reduced BAF content (20%) was used to prepare the virus pools. For the plaque assay well developed monolayer cultures in bottles were used. After introduction of the virus the bottles were kept and occasionally rolled for 30 min at 37C°. The agar overlay, 7.5 ml per 3 oz bottle, consisted of Eagle's medium containing 1.5% Noble Agar to which 10% bovine serum and 10% tryptose phosphate broth were added.

Isolation of virus. The rectal swab was processed on the day it was taken. The fecal material was eluted in 2 ml BSS containing antibiotics, centrifuged, and 0.1 ml of the supernate was inoculated directly on the monolayer sheet. After 30 min incubation maintenance medium was introduced and the tubes kept in a rolling drum at 37°C temperature.

Virus assay. Serial 10-fold dilutions of infected tissue culture fluid were inoculated into 4 tissue culture tubes in 0.1 ml volume. The $TCID_{50}$ was determined by the method of Reed and Muench(9).

Preparation of immune serum. Rhesus monkeys and rabbits were inoculated with concentrated virus suspension using the incomplete adjuvant technic. Each animal received 3 intramuscular 2.5 ml injections, at 3 week intervals. Animals were bled 2-3 weeks after the last injection. The rabbit sera had an antibody titer of 1:240, the monkey 1:2,560 against 100 $TCID_{50}$ virus.

Neutralization test. The standard serological technic used in studies with enteroviruses (*i.e.*, testing of serum dilutions for neutralizing capacity against 100 $TCID_{50}$ virus) was used(10).

Electron microscopy. The virus pool, approximately 1,000 ml, was purified by fluorocarbon treatment(13), then concentrated by dialyzing against polyethylene glycol and the fluorocarbon treatment repeated. Kellenberger's agar method(17) was employed for mounting the virus on collodium membrane, and an RCA Model EMU 3 F electron microscope was used.

Density gradient centrifugation. Cesium chloride 40% w/w density 1.43 was used. To 3.51 ml of this solution 1 ml of virus sample was added and mixed. The material was centrifuged in a Spinco Model L ultracentrifuge with swinging bucket (SW 39) rotor at 30,000 rpm for 18 hours. The samples were collected through a hole in the bottom. The band of virus was easily visible and appeared in the middle of the tube.

Experimental results. Isolation and identification of the virus. Appearance of enterovirus-like CPE was noted on the 3rd day reading in the tubes containing FL monolayer cells only, inoculated with the original specimen

TABLE I. Cross Neutralization Test Conducted in Identification of Hu 39 Virus.

	Results
1. Prototypic immune sera tested against Hu 39 virus	
Coxsackie A Type 1-21, B Type 1-6*	Negative
" " " 22,24†	Not done
Poliomyelitis Type 1-3	Negative
ECHO Type 1-28, E 59 (JV 10)‡	"
Frater, PR 10,17,20,22,28, Hu 780,2080, H 130,136,170	"
Giles, HSO, Pett, C-18, Hu 504, 659	"
2. Prototypic viruses tested against Hu 39 immune serum	
Coxsackie A Type 2,3,7,8,9,10,11,12,13,14,15,16,17,18,20,21,24§	"
" " " 1,4,5,6,19,22	Not done
Poliomyelitis Type 1-3	Negative
ECHO Type 1-28, E 59 (JV 10)	"
Frater, PR 10,17,20,22,28, Hu 780,2080, H 130,136,170	"
Giles, HSO, Pett, C-18, Hu 504, 659	"

* In case of rabbit immune serum 1:15, monkey immune serum 1:25 dilution was used.

† Immune serum was not available for testing.

‡ In case of ECHO Type 4 1:5 diluted immune serum was used, and CF test was also performed with type specific guinea pig immune serum(18).

§ Coxsackie A Type 2,3,8,12 viruses, adapted to primary human amnion culture cells failed to grow in FL human amnion line cells used in this laboratory to propagate viruses not pathogenic for monkey kidney cells. In the case of these viruses, cross neutralization tests were done in human amnion primary cell cultures.

on April 14, 1959. The virus was readily carried through 3 passages in FL cells, and after 3 terminal dilution passages a larger pool was made for the identification. The pool had a relatively low titer, in the range of 10^5 $TCID_{50}$ per ml infected fluid. This has been a consistent finding. Repeated efforts to type the virus in neutralization tests using immune sera prepared against the recognized human enteroviruses failed. Potent monkey immune serum failed to neutralize any of the prototype viruses. Numerous other enterovirus-like agents, isolated in this laboratory and elsewhere, were also tested in cross neutralization tests but no immunological cross reaction was revealed between these and the Hu 39 virus. These viruses were: Frater(2), HSO (1), Giles(3), PR 10, 17, 20, 22, 28(4,5), Thai C - 18(6), and several selected "prototype" viruses, showing similar characteristics, isolated in this laboratory(7). The results are summarized in Table I.

Properties of the virus. The virus shows the typical characteristics of an enterovirus. It is resistant to ether treatment, indicating the absence of essential lipids. Magnesium ions (1M) fully stabilize the virus against heat inactivation at 55°C for 120 min, a general property of enteroviruses(14). The virus multiplies readily in human cells only; it is not pathogenic for suckling mice.

Cytopathogenicity of virus. CPE in FL cell cultures, infected with 100 $TCID_{50}$ of virus appears on the second day. A focus of rounded, shrunken, highly refractile cells forms and in 1-2 days the lesion (Fig. 1, 2) extends to the whole monolayer. The process usually goes on to completion with subsequent detachment of the cells from the surface of the glass. Using high multiplicity of infection, CPE becomes evident by the 7th or 8th hour, and destruction of the culture is complete by 16-18 hours after inoculation. Virus titer found in the supernate is usually low. In stained preparations cell-rounding, pyknosis and karyorrhexis were the characteristic findings as seen in Fig. 3, 4 in a Feulgen-stained preparation. In hematoxylin-eosin stained preparations (Fig. 5, 6) the appearance of an eosinophilic mass was noted as early as 6 hours after infection. This increased in size in the center of the cell, displaced the nucleus, already shrunken to one side of the cell and the basophilic cytoplasm to the periphery of the cell. This type of lesion was found to be common and characteristic of human enteroviruses propagated in cell cultures by Barsky(19). Host range of the virus is apparently limited to human cells. The virus multiplies readily, and produces CPE in human amnion (FL, AV3 lines), HeLa, and primary human kidney cell cul-

tures. Neither multiplication nor CPE was found when the virus was inoculated and passaged in rhesus monkey kidney, testis, squirrel monkey kidney, rabbit kidney, mouse kid-

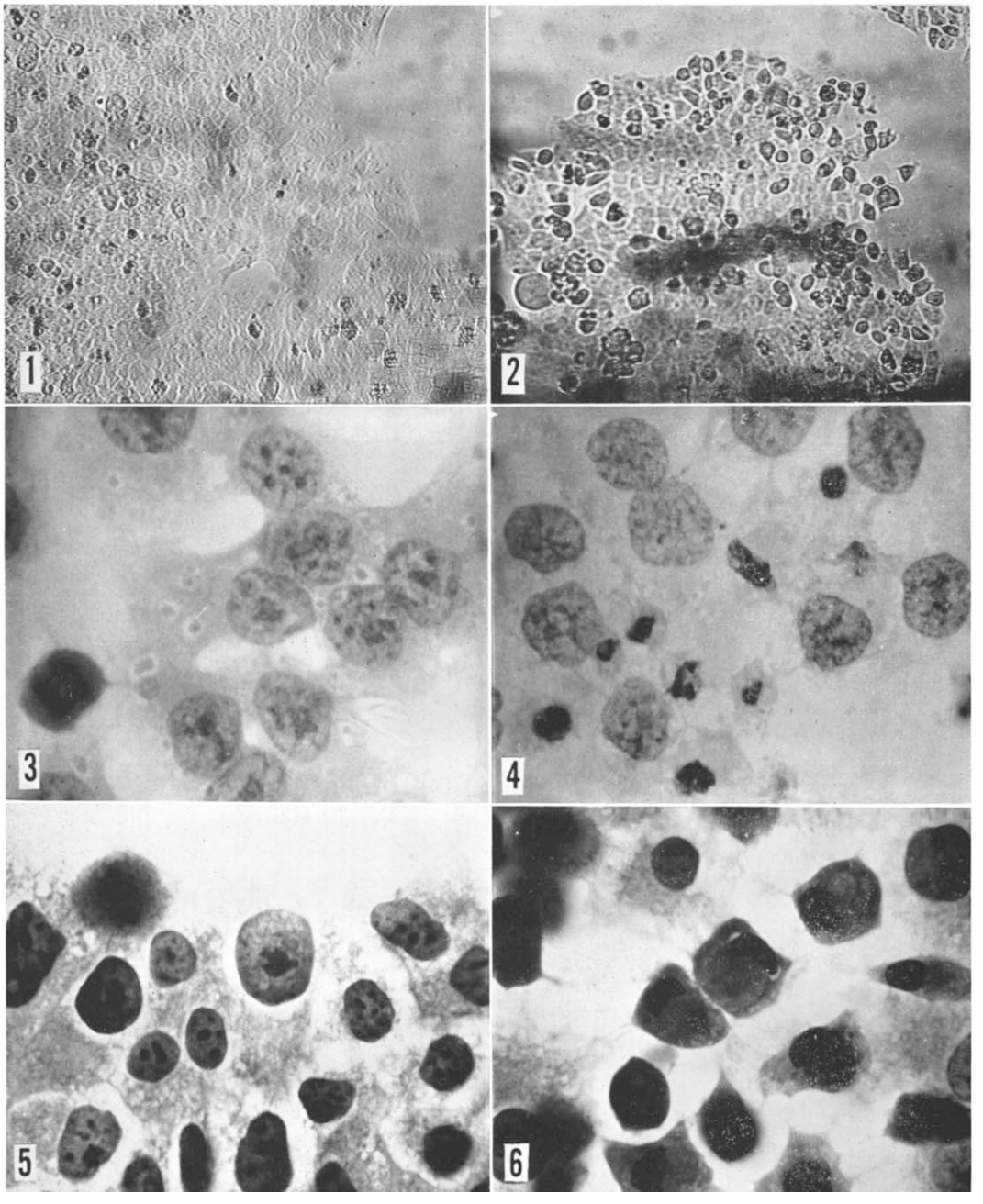


FIG. 1. Normal FL cell cultures. Magnification × 75.

FIG. 2. FL cell cultures infected with Hu 39 virus, 12 hr after infection. Magnification × 75.

FIG. 3. Normal FL cells, Feulgen stained preparation. Magnification × 780.

FIG. 4. FL cells, 12 hr after infection with Hu 39 virus, Feulgen stained preparation. Magnification × 780.

FIG. 5. Normal FL cells, hematoxylin-eosin stained preparation. Magnification × 780.

FIG. 6. Hu 39 virus infected cells, 12 hr after infection. Hematoxylin-eosin stained preparation. Magnification × 780.

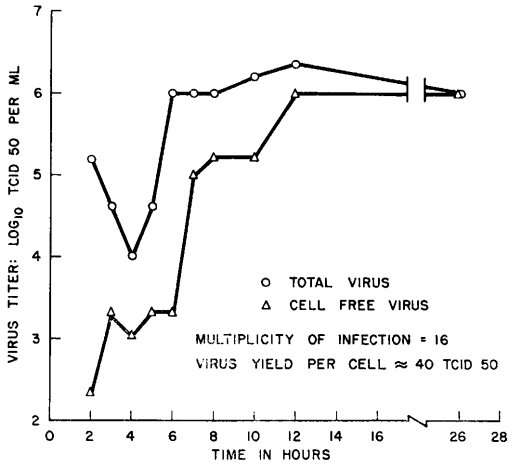


FIG. 7. One step growth curve of Hu 39 virus in FL cells.

ney (adult) or embryonic mouse cell cultures. The virus produced no symptoms of illness in adult mice (i.c., i.p.), suckling mice (s.c., i.p.), guinea pig (i.m., i.p.), rhesus monkey (i.m.) rabbits (i.m., i.v.), inoculation. Chick embryos inoculated intra-allantoically on the 7th day of incubation hatched at the expected time and the birds showed no visible abnormality.

Plaque formation. The virus forms very small, round, hazy plaques on FL monolayer cell cultures, which become visible on the 5th day. Small size and late appearance of the plaques have made technic difficult for routine use.

Multiplication of Hu 39 virus. A one step growth curve experiment revealed growth characteristics similar to those of other enteroviruses. The experiment was done using FL cells in tubes as monolayer cell cultures. An inoculum of high titered virus achieved a multiplicity of infection of 16 and insured that most of the cells became infected. After 90 min for adsorption the tubes were decanted and rinsed 3 times with 5 ml of Hanks' BSS to eliminate non-adsorbed virus. One ml of Eagle's medium—BAF was introduced (pH 7.8) into each tube as maintenance medium. At intervals 5 tubes were withdrawn and virus content of supernatant and of total virus was determined. Intracellular infectious virus began to appear after a retarded eclipse phase of about 5 hours duration (Fig. 7). After approximately 2 hours intracellular virus titer reached a plateau, which was maintained by release of virus from cells infected later. Appearance of extracellular virus exhibited a similar rise approximately 2 hours later. As late as 10 hours after infection, most of the virus (circ. 90%) was still found within the cells. Virus yield per cell was found to be very low: approximately 40 $TCID_{50}$ per infected cell. This low yield is explained partly by the marked heat sensitivity of the virus.

Morphology of virus particle. The size of the virus particle was determined by ultrafiltration through graded millipore filter discs (Millipore Filter Corp.). No loss in titer was noted following passage through a 300 mμ filter, but 90% of the virus was held back by a 50 mμ filter and 100% by one of 10 mμ pore diameter. These data suggest a particle size larger than 10 mμ but smaller than 50 mμ. Extrapolation of the curve gave a particle size of 25-30 mμ. An electron micrograph of air dried preparations revealed the presence of uniform round particles (Fig. 8). Average diameter of the round particles was found to be 34.15 mμ ± 5.2 mμ. This value is very close to those established for poliomyelitis and other enteroviruses.

Hemagglutination. The hemagglutinating activity of the agent was tested by the method of Goldfield(15) at 37°C, room and ice box temperature. No hemagglutination was observed using chick, monkey, rabbit, guinea pig, rat, sheep, human Type O, and bovine erythrocytes. A low hemagglutination titer (1:8) was found using mouse red blood cells,

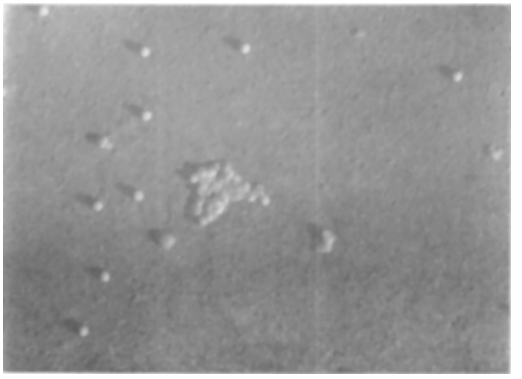
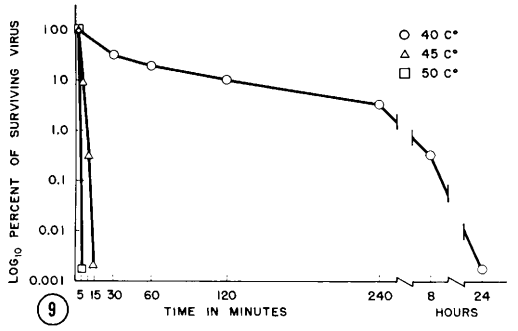
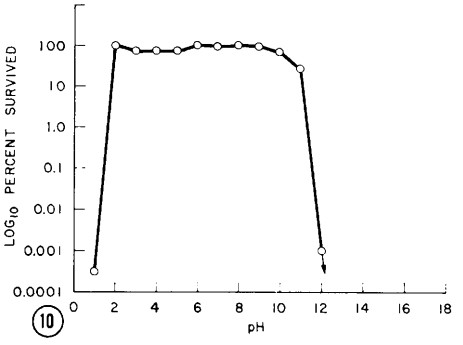


FIG. 8. Electron micrograph of an air dried preparation of Hu 39 virus. Magnification × 32,160; shadowed with platinum-palladium.





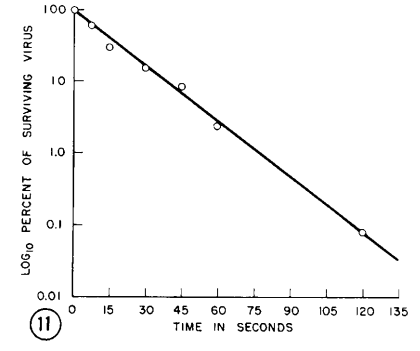


FIG. 9. Heat inactivation of Hu 39 virus.
FIG. 10. Stability of Hu 39 virus at different pH.
FIG. 11. Inactivation of Hu 39 virus by ultraviolet light.

but only at a temperature of 4°C.

Stability. Heat inactivation experiments were carried out using concentrated virus in tightly stoppered Wassermann tubes which were submerged in water baths at various temperatures. Samples were withdrawn at regular intervals, chilled in crushed ice, and amount of surviving virus determined (Fig. 9). The agent was found to be very sensitive to heat inactivation. In this respect it appears to be more sensitive than other enteroviruses. Thus the virus was rapidly inactivated even at temperatures as low as 40°C. Resistance to inactivation by low or high concentrations of hydrogen ions, on the other hand, was found to be quite comparable to other enteroviruses. The virus was found to be stable within pH range of 2 to 11, while it was very rapidly and completely inactivated at lower or higher pH values—when tested at room temperature for 30 minutes (Fig. 10). The virus was readily inactivated by ultraviolet light (Fig. 11) using a standard germicide lamp and irradiating a thin layer of virus from a distance of 82 cm. The curve indicates single hit inactivation of the virus particles.

Nature of the virus. Indirect evidence has indicated that the virus is an RNA virus. 5-bromodeoxyuridine (10 γ/ml), 5-fluorodeoxyuridine (5 γ/ml) or aminopterin (10 γ/ml) failed to inhibit the multiplication of Hu 39 virus in FL cells exposed for 8 hours to these compounds prior to inoculation. The compounds inhibited multiplication of vaccinia virus, which could be reversed by addition of thymidine (50 γ/ml). Even higher concentrations of the compounds (up to 50-fold) failed to interfere with multiplication of Hu 39 virus. When the pellet of concentrated and CsCl isodensity centrifuged purified virus was stained with acridine orange according to the method of Mayor(16), orange colored fluorescence was observed, indicating presence of RNA.

The density of the virus in CsCl isodensity centrifugation was compared with that of poliomyelitis virus. When the radioactivity of P^{32} labelled poliomyelitis virus and the infectivity of the Hu 39 virus were determined in each fraction, the results indicated identical densities.

The sedimentation coefficient was determined in Spinco Model E analytical ultracentrifuge by Dr. F. Lamy, Department of Anatomy, University of Pittsburgh School of Medicine, to whom we are indebted. The coefficient 159.75×10^{-13} for Hu 39 virus is comparable to that of poliovirus.

Prevalence of Hu 39 virus. The virus was isolated only from a single stool specimen. In subsequent years we examined 2275 stool specimens obtained from 516 children in the

neighborhood of Pittsburgh. Hu 39 was not recognized among the 209 virus strains isolated. Attempt was made to demonstrate presence of antibodies in the blood of people residing in Allegheny County (or Western Pennsylvania). Of 277 adult sera tested, antibody against the virus was found in only one at the screening dilution level of 1:8. The serum exhibited a neutralizing titer of 1:64 against 175 $TCID_{50}$. These data indicate a very limited prevalence of the virus in the population of Pittsburgh during the last few years. Human gamma globulin diluted 1:20 failed to neutralize the virus. The presence of specific antibodies in human serum in one instance and the fact that the virus was successfully reisolated from the original specimen in 1962 indicate that the agent is very likely a human enterovirus. The possible relationship of the virus to the clinical disease observed in the patient from whom the agent was isolated could not be investigated in the absence of acute and convalescent serum specimens.

Summary. A virus was isolated in human amnion FL cell cultures on April 14, 1959 from the rectal swab obtained from a 9-month-old male child admitted to the Children's Hospital, University of Pittsburgh, with an acute respiratory illness of undetermined etiology. The virus exhibits the properties of a typical enterovirus. It is not pathogenic for suckling mice, and multiplies and produces CPE only in human cells. It was not isolated in subsequent years from 2275 rectal swabs obtained from 516 children. Serological studies in a normal population group in the Pittsburgh area revealed antibodies against the virus in one serum among 277 adult sera tested. This fact and the re-isolation of the virus from the original specimen suggest that it is a human enterovirus. No conclusion can be drawn concerning its pathogenicity for man.

The expert technical assistance of Mrs. Christine Ziegler is gratefully acknowledged.

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Effect of Ouabain on Cardiac Output of Anesthetized Rat.* (28159)

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Albino rats are considered resistant to ouabain. Large doses are required to produce electrocardiographic changes indicative of toxicity in this species(1). The relationship

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