Although it was known that reoviruses of human origin could infect cattle, this is the first demonstration that a reovirus of animal origin can infect man. It has not yet been demonstrated that reoviruses are naturally transmitted from lower animals to man, or vice versa; however, the sum of the presently available evidence suggests that such an event is not unlikely.

Summary. Inoculation of volunteers with a bovine strain of reovirus type 1 produced infection in adult human volunteers. Following intranasal administration of the agent, virus excretion, serologic response and lack of clinical manifestations were similar to those observed in volunteers experimentally infected with a reovirus type 1 strain of human origin.

1. Rosen, L., *Ann. N. Y. Acad. Sci.*, 1962, in press.
2. ———, *Am. J. Hyg.*, 1960, v71, 250.
3. Eagle, H., *Science*, 1955, v122, 501.
4. Kravetz, H. M., Knight, V., Chanock, R. M., Morris, J. A., Johnson, K. M., Rifkind, D., Utz, J. P., *J.A.M.A.*, 1961, v176, 657.
5. Reed, L. J., Muench, H., *Am. J. Hyg.*, 1938, v27, 493.
6. Rosen, L., *ibid.*, 1960, v71, 242.
7. Rosen, L., Evans, H. E., Spickard, A., *ibid.*, 1963, in press.

Received January 3, 1963. P.S.E.B.M., 1963, v112.

Virus Isolation Studies of Stool Specimens Obtained from Patients With Cholera.* (28227)

ANDREW S. ABRAHAM AND FRANCIS S. CHEEVER
Department of Microbiology, University of Pittsburgh School of Medicine, Pittsburgh, Pa.

The 1958-1959 cholera outbreak in Bangkok, Thailand, was studied by several groups of investigators from the U.S.A.(1,2,3,4) and an account of the viral isolation studies was given by Johnston *et al.*(4). Enteroviruses of recognized serotypes were occasionally isolated. No etiological relationship was found between the enteroviruses isolated and the clinical disease. We also had the opportunity of studying the problem of the association of enteroviruses with cholera(5) and our results are in full agreement with those of Johnston *et al.* Although 4 enteroviruses were isolated, we found no significant association between them and the clinical disease. Besides known enteroviruses, we have isolated a virus (Thai C-18) similar in behavior to the Coxsackie A group of viruses, but not identical with any of the recognized human enterovirus serotypes. The properties of Thai C-18 virus are given in this report.

Materials and general methods. Source of materials. 40 Fecal specimens were secured through the kind cooperation of Drs. K. Goodner, R. Freter and Harry Smythe, Jefferson Medical College, Philadelphia. We received also 17 additional stool specimens through the kindness of Col. Felsenfeld and his associates at SEATO cholera laboratory. The specimens obtained by the Jefferson team were collected at a time when the epidemic was on the wane, and some patients were no longer in the acute stage of the clinical disease. The SEATO laboratory specimens were secured some months later during an apparent recrudescence of the same outbreak. All specimens were frozen promptly and sent, packed in dry ice, to Pittsburgh. Paired sera for antibody studies were obtained from as many patients as possible. Bacteriological studies were carried out in Bangkok and the results made available to us. Although a clinical diagnosis of cholera was made in each instance, some of the cases were regarded as atypical. The *Vibrio comma* isolation rate was approximately 63%.

Source of viruses and immune sera. Poliomyelitis Type 1-3, Coxsackie A Type 7, 9, B Type 1-6, ECHO Type 1-27 viruses were

* This investigation was carried out under the sponsorship of the Commission on Enteric Diseases of Armed Forces Epidemiological Board, and was supported by Office of the Surgeon General, Dept. of the Army.

kindly supplied by Dr. D. S. Yohn; ECHO Type 28 virus and immune serum by Dr. W. J. Mogabgab; E 59 virus (JV 10) by Dr. W. C. Branche; Coxsackie Type 21 (Coe) by Dr. E. H. Lennette; Coxsackie Type 24 (Pett) virus by Dr. J. A. Kasel; and Coxsackie A Type 2, 3, 8, 11, 12, 13, 14, 15, 16, 17, 23 viruses adapted to primary human amnion culture cells by Dr. H. A. Wenner(25). Some of the non-prototype, but enterovirus-like viruses were also tested for identification. HSO virus and immune serum(6) were kindly supplied by Dr. M. Matumoto; the Frater virus (7) by Dr. P. Kamitsuka; the Giles virus and rabbit immune serum(8) by Dr. M. Cooney; the PR 10, 17, 20, 22, 28 viruses and rabbit immune sera(9,10) by Dr. W. C. Branche. Several other viruses, isolated in this laboratory, were also tested(11,12,13). Immune sera, prepared in rabbits against Poliomyelitis Type 1-3, Coxsackie B Type 1-5, ECHO Type 1-6, 8-10, 12-15 were purchased from Microbiological Associates, Inc., Bethesda, Md. Coxsackie A Type 1-19, ECHO Type 7, 11, 16-25 immune sera prepared in monkeys were furnished by Dr. J. T. Syverton; Coxsackie B, Type 6, ECHO Type 26, 27 monkey immune sera were donated by Dr. D. S. Yohn; ECHO Type 28 rabbit immune serum was given by Dr. W. J. Mogabgab. Immune sera against Coxsackie A Type 20, 20/a, 21 (Coe), 24 (Pett), HSO virus and the viruses isolated by us were prepared in monkeys in this laboratory, with the exception of virus Hu 2220 which was prepared in roosters.

Tissue cultures. Trypsin dispersed monkey kidney cell cultures were prepared by a minor modification of Youngner's method(14). The maintenance of human amnion FL cell line (15) cultures and preparation of tube and bottle cell cultures were described previously (13); the only difference was the use of M 199 instead of Eagle's medium.

Virus isolation. The fecal material, of fluid consistency, was treated with antibiotics and, after centrifugation for 30 min at 2,500 rpm, was inoculated directly in well developed monolayer human amnion (FL line) and primary kidney cell culture tubes, in 0.2 ml volume. After 30 minutes of incubation at 37C° temperature, maintenance medium was introduced and the tubes were kept in a roller drum at 37C° temperature for 2 weeks. The tubes were checked for the appearance of CPE every other day. Three blind passages were carried out in the case of FL cell cultures, 2 in the case of monkey kidney cell cultures. One litter of suckling mice was inoculated, within 24 hours of birth, with each specimen. Each mouse received 0.05 ml s.c. and the animals were observed for 2 weeks. One blind passage was carried out by injection of a suspension of ground up carcasses.

Virus assay. Serial 10-fold dilutions of infected cell culture fluid were inoculated into 4 cell culture tubes in 0.1 ml volume. The $TCID_{50}$ was determined by the method of Reed and Muench(16).

Preparation of immune serum. Rhesus monkeys and rabbits were inoculated by injection of partially purified and concentrated virus suspension, using the incomplete adjuvant technique. Each animal received 3 intramuscular 2.5 ml injections, at 3 week intervals. The animals were bled 2-3 weeks after the last injection. The rabbit sera had an antibody titer of 1:80, the monkey sera 1:5,120 when tested against approximately 100 $TCID_{50}$ virus.

Neutralization test. The standard serological technique used in studies with enteroviruses (*i.e.*, testing of serum dilutions for neutralizing capacity against approximately 100 $TCID_{50}$ virus) was used(17).

Electron microscopy. The virus pool, approximately 1,000 ml, was purified by fluorocarbon treatment(18), then concentrated by dialyzing against polyethylene glycol (Carbowax 6,000), and the fluorocarbon treatment above was then repeated twice. Kellenberger's agar method(19) was employed for mounting the virus on collodium membrane. A Philips Model 200 electron microscope was used for taking the electron micrographs, through the kind help of Dr. Leonard Napolitano (Department of Anatomy).

Isolation and identification of the viruses. No virus was isolated in monkey kidney culture cells. Four viruses were isolated in human amnion (FL line) culture cells. The presence of significant CPE could not be established in the first passage, as the presence

TABLE I. Cross Neutralization Test Conducted in Identification of C-18 Virus.

	Results
1. Prototypic immune sera tested against C-18 virus	
Coxsackie A, Type 1-12, B, Type 1-6*	Negative
Coxsackie A, Type 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, 2.080, H 130, 136, 170	"
Giles, HSO, Pett, Hu 39, 504, 659, 2.220	"
2. Prototypic viruses tested against C-18 immune serum	
Coxsackie A, Type 2, 3, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 20, 21,§ B, Type 1-6	"
Coxsackie A, Type 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, 2.080, H 130, 136, 170	"
Giles, HSO, Pett, Hu 39, 504, 659, 2.220	"

* In case of rabbit serum 1:15, monkey 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(24).

§ Coxsackie A, Type 2, 3, 8, 12 viruses, adapted to primary human amnion culture cells(25), 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.

of proteolytic enzymes derived from the intestinal tract induced rounding and sloughing off the cells from the wall of the tube. The CPE induced by these viruses were readily observable in the second passage. One virus, the Thai C-18 was isolated from one of the first 40 specimens, 3 others (NC 6, 7, 11) from 3 different members of the second 17 specimens. None of them proved to be pathogenic for monkey culture cells, but all were found to be pathogenic for suckling mice. Three agents of the 4 (NC 6, 7, 11) were also isolated in suckling mice, but the Thai C 18 was not recovered by this method. No other viruses were isolated in suckling mice. All 4 viruses were carried through 4 terminal dilution passages before identification was attempted. Three viruses (NC 6, 7, 11) were neutralized by immune serum prepared against Coxsackie A, Type 18 virus, and the immunological relationship proven by cross neutralization tests. The fourth virus, Thai C 18, showed no immunological cross reaction with any of the recognized human enteroviruses. The data on the identification procedures are summarized in Table I.

Serological studies. A number of paired sera from patients was available to us, as were also a few unmatched acute phase serum specimens. Antibody studies were carried out with the 4 viruses isolated in an effort to demonstrate the rise of antibody titer indicating the concurrent infection with these viruses. The results are summarized in Table II. In addition, a few of the unmatched acute phase serum specimens showed low antibody titers (1:8 or 1:16) against one or more of these viruses.

Properties of C-18 virus. The general characteristics of the virus resembled those of the Coxsackie A Group viruses. The virus was resistant to ether treatment, indicating the absence of essential lipids in the virus. Magnesium ions in 1 Molar concentration fully stabilized the virus against heat inactivation by 55°C for 120 minutes, a general characteristic of human enteroviruses(20).

Pathogenicity of C-18 virus. The CPE in FL cell cultures infected with 100 $TCID_{50}$ virus dose appeared on the second day. Foci of rounded, shrunken, highly refractile cells were first noted (Fig. 1), and in a few days the lesion extended to the whole monolayer cell culture. The cytopathogenic process usually went on to completion with subsequent detachment of the cells from the surface of the glass into the medium. Using high multiplicity of infection, the CPE became evident by the sixth or seventh hour, and the destruction of the cell culture was complete by approximately 12 hours after in-

TABLE II. Prevalence of Antibodies in Paired Sera of Cholera Patients.

		Antibody status		
		Present in both sera		
Viral isolate	No. of paired sera tested	Significant rise in titer	No change in titer	Absent in both sera
C-18	36	1	1	34
NC- 6	36	1	1	34
NC- 7	36	4*	6	26
NC-11	36	1	2	33

* One of these represented the patient from the virus (NC-7) was isolated.

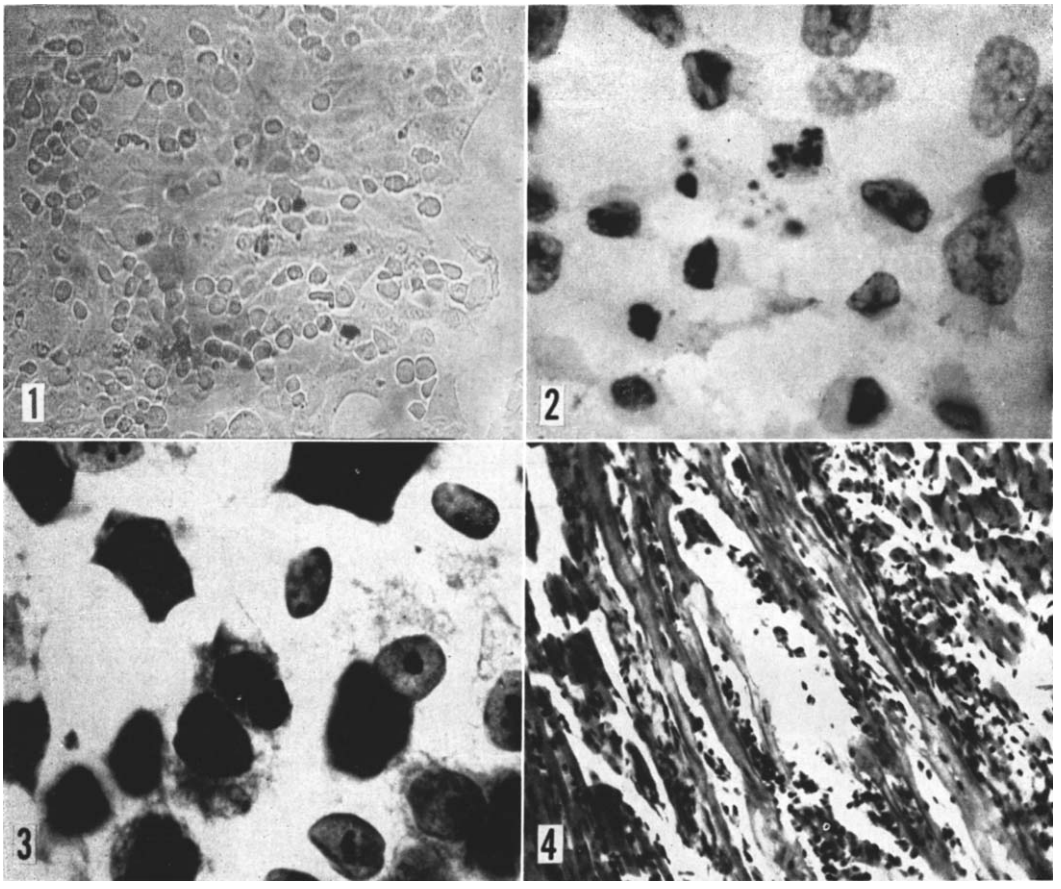

FIG. 1. FL cell cultures infected with C-18 virus, 12 hr after infection. Magnification ×111.
FIG. 2. FL cells, 12 hr after infection with C-18 virus, Feulgen stained preparation. Magnification ×780.
FIG. 3. FL cells, infected with C-18 virus, 12 hr after infection. Hematoxylin-eosin stained preparation. Magnification ×780.
FIG. 4. Inflammatory lesion and hyaline degeneration of muscle caused by C-18 virus in suckling mice. Right front leg, 3 days after inoculation, hematoxylin-eosin stained preparation. Magnification ×225.

fection. The virus titer found in the supernatant was usually high (10^7 PFU/ml). In stained preparations (Giemsa, Methyl-Green Pyronin, Feulgen, Acridine Orange), the rounding of the cells was associated with pyknosis and karyorrhexis (Fig. 2) similar to the CPE produced by other enteroviruses. In hematoxylin-eosin stained preparations (Fig. 3) the appearance of a centrally located eosinophilic mass was noted approximately 6 hours after infection. As this increased in size, it displaced the cytoplasm and the already shrunken nucleus to the periphery of the cell. The virus multiplied readily and produced similar types of CPE in human amnion primary, FL and AV_3 lines, HeLa, and primary human kidney cell cultures. No CPE was found when the virus was inoculated and passaged in rhesus monkey kidney, or testis, squirrel monkey kidney and rabbit kidney cell cultures. The virus produced no sign of illness in adult mice (i.c., i.p.), guinea pig (i.m., i.p.), rhesus monkey (i.m.), rabbits (i.m. i.p.) inoculation. Chick embryos, inoculated on the 7th day of incubation, hatched at the expected time and the birds showed no visible abnormality. When high titered virus was inoculated into suckling mice (0.005 ml s.c.), severe paralysis of the extremities was noted and death occurred in some of the animals,

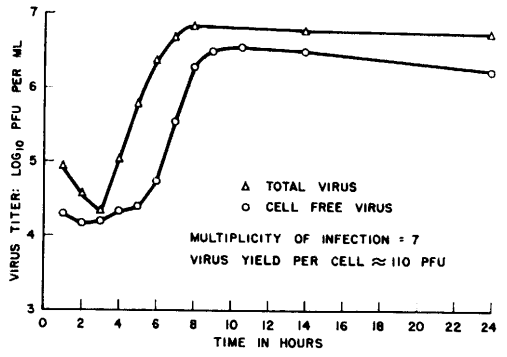

FIG. 5. One step growth curve of C-18 virus in FL cells.

while no clinical sign was noted with others. The signs of paralysis of the extremities appeared on the 3rd-5th day, and death, when it occurred, came a few days later. Histological section of the extremities involved showed signs of inflammation and severe degeneration of muscle fibers, a picture similar to that found with the Coxsackie A group of viruses (Fig. 4). Several attempts to passage the virus in suckling mice were unsuccessful.

Plaque formation of C-18 virus. The virus formed clear, round plaques, approximately 5-7 mm in diameter, on FL monolayer cell cultures. The plaques appeared 3-4 days after inoculation.

Multiplication of C-18 virus. Adsorption of the virus to the cell was rapid, at 37°C. A one-step growth curve experiment revealed growth characteristics similar to those of human enteroviruses (poliomyelitis virus). The experiment was done using FL cells as monolayer cell cultures. An inoculum of high titered virus achieved a multiplicity of infection of 7 and insured that most of the cells became infected. After 90 minutes of adsorption the fluid was decanted and the tubes rinsed 3 times with 5 ml of Hanks' BSS to eliminate the non-adsorbed virus. One ml of M 199-BAF (pH 7.8) was introduced into each tube as maintenance medium. At intervals 5 tubes were wtihdrawn and the virus content of the supernatant and of the total virus was determined (Fig. 5). After a short eclipse phase the intracellular infectious virus began to appear about 4 hours after infection. After approximately 2 hours the intracellular virus reached a plateau, which was further maintained by the synthesis of virus by cells infected later. The titer of extracellular virus exhibited a similar rise approximately 2 hours later, reflecting the release of virus from the cells. Most of the virus was found within the infected cells. The virus yield per cell was approximately 100 PFU per cell.

Morpology of virus particle. The size of the virus was determined by ultrafiltration through graded millipore filter discs (Millipore Filter Corp., Bedford, Mass.) Extrapolation of the curve of transmitted virus *vs* pore size (average) indicated a particle size approximately 20 mμ. Pictures of air dried preparations taken by the electron microscope revealed the presence of uniform round particles (Fig. 6). The average size of the particles was found to be 27.2 mμ ± 3.52 mμ, which falls in the range of poliomyelitis and other enteroviruses.

Hemagglutination. The hemagglutinating activity of the virus was tested by the method of Goldfield(21) at 37°C, room, and 4°C temperature. No hemagglutination was observed using chick, rhesus monkey, guinea pig, rat, sheep, human Type O, mouse, bovine red blood cells. In case of rabbit red blood cells a low hemagglutination titer of 1:8 was found, but only at 4°C temperature.

Stability of virus. Heat inactivation experiments were carried out, using high titered virus placed in tightly stoppered Wassermann tubes and submerged in water baths at various temperatures. Sets of samples were withdrawn at intervals, chilled in crushed ice, and amount of surviving virus determined (Fig. 7). The virus was moderately heat sensitive, similar to some ECHO viruses(22) but less resistant than some poliomyelitis virus strains (23). Resistance of the virus against inactivation by H^+ and OH^- ions was found to be similar to that found with other enteroviruses. The virus was found to be stable within a pH range of 2 to 11, while it was rapidly inactivated at lower or higher pH values, when tested at room temperature for 30 minutes.

Nature of virus. Indirect evidence indicated that the virus is an RNA virus. 5-Bromodeoxyuridine (10/ml), 5-fluorodeoxyuridine (5/ml) or aminopterin (10/ml) failed

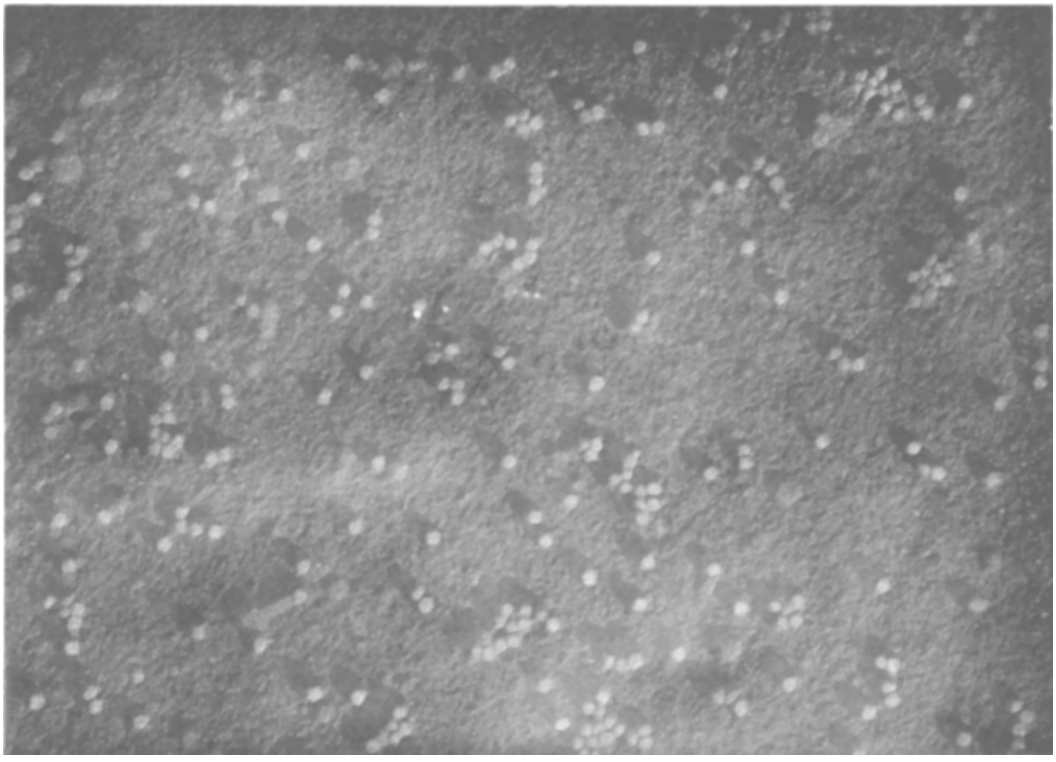

FIG. 6. Electron micrograph of an air dried preparation of C-18 virus. Magnification ×27,658. Shadowed with Platinum - Palladium.

to inhibit the multiplication of C-18 virus in FL cells pretreated for 8 hours by these inhibitors. These compounds successfully inhibited the multiplication of vaccinia virus, which inhibition process could be reversed by addition of thymidine (50/ml).

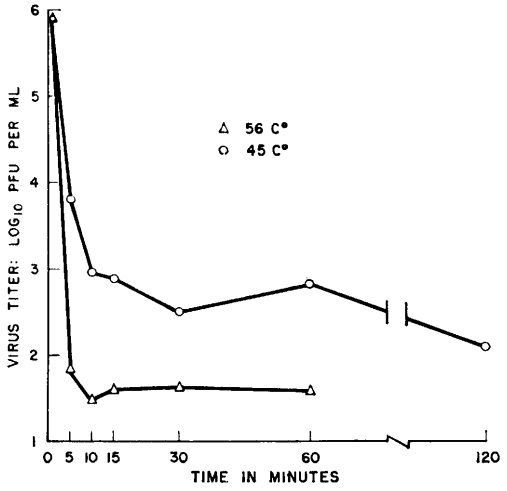

FIG. 7. Heat inactivation of C-18 virus.

Prevalence of C-18 virus. As pointed out, neutralizing antibody was found in 2 of 36 sera cholera patients hospitalized in Bangkok, Thailand (Table II). A representatvie sample of sera (110) from normal adults resident in the Pittsburgh area yielded no evidence for the presence of neutralizing antibody. Similarly, gamma globulin of American origin yielded negative results. These data indicate that the virus is probably not present in the Pittsburgh area.

Discussion. As other reports on the cholera epidemic in Bangkok in 1959 have indicated(1,2,3,4), the clinical diarrheal disease was mild, the case fatality rate was low, and the *Vibrio comma* isolation rate was approximately 63%. Of the 57 stool specimens obtained from cholera patients, only 4 viruses were isolated. Three of these showed immunological relationship to the Coxsackie A Type 18 virus. There was no evidence that either these or the fourth virus (C-18, not identical with any of the recognized human enteroviruses,) played any role in the disease.

The patient, coded as C-18 was a 58-year-old woman, admitted on March 10, 1959, with a complaint of diarrhea (which began 6 hours before admission) unaccompanied by nausea or vomiting. She had been vaccinated against cholera 7 days previously. Physical examination revealed evidence of mild dehydration; the blood pressure was not recorded. The patient staged an uneventful recovery. Fecal material for viral studies was obtained on the day of admission. No cholera vibrios were isolated in spite of repeated stool cultures. Serum specimens were obtained on the day of admission and 16 days later. No antibody against C-18 virus was detected in either the acute or convalescent serum specimen. When paired sera (acute and convalescent) collected from 36 cholera patients were examined for antibody against the C-18 virus, only in one patient was a rise of antibody titer detected during the illness. In another patient both the acute and convalescent serum specimens showed the same titer (1:16). These findings yield no evidence to suggest that a viral agent (or agents) plays a significant role in the pathogenesis of cholera. The viral isolation rate was low (less than 10%), and the antibody responses when paired sera were tested of borderline significance. These negative results are in accord with the findings reported by Johnston *et al.*(4). Of theoretical interest is the fact that a virus, similar to the human enteroviruses, but not identical with any of the recognized human enterovirus serotypes, was isolated.

Summary. No evidence has been obtained from the 1959 outbreak of cholera in Bangkok, Thailand that a viral agent played a significant role in the pathogenesis of the diarrheal disease observed. From one patient an enterovirus (Thai C-18) was isolated similar to the Coxsackie A Group of viruses which was not identical with any of the recognized human enteroviruses. Some of the properties of this agent have been described.

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

1. SEATO Conference on Cholera, Bangkok, SEATO, 1962.

2. Siddhichai, P., Grayston, J. T., *Am. J. Hyg.*, 1960, v72, 149.

3. Morgan, F. M., Felsenfeld, O., Rodvatanakul, B., Buspavanich, S., Bandhumedha, B., Chowvanasai, A., *ibid.*, 1960, v72, 250.

4. Johnston, P. B., Grayston, J. T., PROC. SOC. EXP. BIOL. AND MED., 1960, v104, 293.

5. Cheever, R. S., Abraham, A. S., In SEATO Conference on Cholera: papers presented and summaries of discussions, Dec., 1960. Bangkok, SEATO, 1962.

6. Matumoto, M., Muati, M., Aoyama, Y., *Jap. J. Exp. Med.*, 1960, v30, 213.

7. Duncan, I. B. R., *Lancet*, 1960, v2, 470.

8. Cooney, M. K., McLaren, L. C., Bauer, H., *Am. J. Hyg.*, 1962, v75, 301.

9. Branche, W. C., Young, V. M., *Fed. Proc.*, 1961, v20, 440.

10. ———, *Bact. Proc.*, 1962, 136.

11. Abraham, A. S., Cheever, F. S., *ibid.*, 1961, 146.

12. ———, *ibid.*, 1962, 160.

13. Abraham, A. S., PROC. SOC. EXP. BIOL. AND MED., 1963, submitted for publication.

14. Youngner, J. S., *ibid.*, 1954, v85, 202.

15. Fogh, J., Lund, O. R., *ibid.*, 1957, v94, 532.

16. Reed, L. J., Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

17. Diagnostic Procedures for Virus and Rickettsial Diseases, *Am. J. Publ. Health Assn.*, 1956, p. 80.

18. Manson, L. A., Rothstein, E. L., Rake, G. W., *Science*, 1957, v125, 546.

19. Kellenberger, E., Arber, W., *Virology*, 1957, v3, 245.

20. Wallis, C., Melnick, J. L., *ibid.*, 1962, v16, 504.

21. Goldfield, M., Srihongse, S., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 788.

22. Lehman-Grube, F., Syverton, J. T., *Am. J. Hyg.*, 1959, v60, 161.

23. Youngner, J. S., *J. Immunol.*, 1957, v78, 282.

24. Yohn, D. S., Hammon, W. McD., PROC. SOC. EXP. BIOL. AND MED., 1960, v105, 55.

25. Wenner, H. A., Lenahan, M. F., *Yale J. Biol. Med.*, 1961-62, v34, 421.

Received January 7, 1963. P.S.E.B.M., 1963, v112.