1. Rasmussen, H., DeLuca, H. F., Ergeb. Physiol. Biol. Chem. Expt. Pharmakol., 1963, v33, 109.
2. Wasserman, R. H., Ed., The Transfer of Calcium and Strontium Across Biological Membranes, Academic Press, New York, 1963.
3. Eisenstein, R., Passavoy, M., Proc. Soc. Exp. Biol. Med., 1964, v117, 77.
4. Zull, J. E., Czarnowska-Misztal, E., DeLuca, H. F., Science, 1965, v149, 182.
5. Norman, A. W., ibid., 1965, v149, 184.
6. Schachter, D., Kowarski, S., Bull. N. Y. Acad. Med., 1965, v41, 241.
7. Norman, A. W., Biochem. Biophys. Res. Comm., 1966, v23, 3, 335.
8. Harrison, H. E., Harrison, H. C., Proc. Soc. Exp. Biol. Med., 1966, v121, 312.
9. Taylor, A. N., Wasserman, R. H., Nature, 1965, v205, 248.
10. Wasserman, R. H., Taylor, A. N., ibid., 1963, v198, 30.
11. Taylor, A. N., Wasserman, R. H., Arch. Biochem. Biophys., in press.
12. Wasserman, R. H., Taylor, A. N., Science, 1966, v152, 791.
13. Thomas, W. C., Jr., Morgan, H. G., Connors, R. B., Haddock, L., Bills, C. E., Howard, J. E., J. Clin. Invest., 1959, v38, 1078.
14. Briggs, F. N., Fleischman, M., J. Gen. Physiol., 1965, v49, 131.
15. Bray, G. A., Anal. Biochem., 1960, v1, 279.
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., J. Biol. Chem., 1951, v193, 265.
17. Wasserman, R. H., Taylor, A. N., Kallfelz, F. A., Am. J. Physiol., 1966, v211, 419.

Received December 22, 1966. P.S.E.B.M, 1967, v125.

Incompetent Relationship Between Coxsackievirus A6 Ribonucleic Acid and Mouse Cells.* (32012)

JOSEPH W. ST. GEME, JR.,† PATRICIA S. TOYAMA, JOSEPHINE L. BRUMBAUGH, AND MONICA E. DIETZ (Introduced by D. T. Imagawa)
Department of Pediatrics, University of Minnesota Medical School, Minneapolis, Minn. and University of California School of Medicine, Los Angeles

Previous work(1) established the paradoxical insusceptibility of primary and stable mouse cells *in vitro* to coxsackievirus A6, an enterovirus lethal for newborn mice *in vivo*. It was suggested that cultivation of mouse cells *in vitro* may alter viral receptors or disrupt intracellular mechanisms for synthesis of this virus.

Subsequently Came and Crowell(2), employing coxsackieviruses A13 and A18 capable of quantitation by plaque assay, demonstrated that the resistance of mouse fetal cells *in vitro* to these enteroviruses may be explained by loss of viral receptors. These investigators studied viral attachment to cultivated cells in order to elucidate this biologic paradox.

Kinetic studies of viral attachment to host cells or tissues necessitate precise quantitation with which strains of coxsackievirus A6 may be obtained only by newborn mouse bio-assay *in vivo*. In order to resolve the issue of altered viral receptors, primary mouse fetal cells were exposed to RNA extracted from the test virus, coxsackievirus A6, and a positive control virus, mengovirus, capable of multiplication and cytopathogenicity in cultivated mouse cells. The role of receptors in this host cell-virus relationship was further clarified by the study of coxsackievirus A6 replication in suspensions of non-trypsinized, minced, mouse fetal tissue. The biologic integrity of the preparations of viral RNA was assessed by intracerebral inoculation of susceptible newborn mice.

Materials and methods. Viruses. Coxsackievirus A6, strain C. G. (Gdula), was obtained from the American Type Culture Collection (#VR 165) as a 20% suspension of newborn mouse (NM) torso and brain and passed in-

* This investigation was aided by grants from the Minnesota Division, Am. Cancer Soc., and the Attending Staff Association, Harbor General Hospital.

† Present address: Dept. of Pediatrics, Harbor General Hospital, Torrance, Calif., U.C.L.A. School of Med.

traperitoneally (IP) in Swiss albino newborn mice. The eviscerated torso was harvested subsequent to hind limb paralysis and death, prepared as a 10% suspension in maintenance medium (MM) consisting of 5% heat-inactivated calf serum and 95% Eagle's basal amino acid and vitamin supplement (E) in Hanks' balanced salt solution (BSS), distributed into glass ampules, fire-sealed, and frozen at —50°C. The 10% torso suspension of stock virus was assayed by IP inoculation of 0.05 ml volumes of serial 10-fold dilutions per litter of 1-4 day old newborn mice. The 50% lethal dose (LD_{50}) endpoint was determined to be $10^{8.3}$ NM IP LD_{50} per ml. The titer was essentially the same by intracerebral (IC) inoculation of 0.01 ml volumes.

Mengovirus, obtained *via* Dr. R. W. Hinz from Dr. E. L. Buescher as the 25th mouse brain passage of the original mosquito isolate (3), was passed IC in Swiss albino weanling mice, the brain harvested subsequent to illness and death, prepared as a 10% tissue suspension in MM, and distributed and frozen as above. The 10% brain suspension of stock virus was assayed in replicate slanted tube cultures of mouse fetal cells by inoculation of 3 cultures with 0.1 ml of each serial 10-fold dilution in MM. The 50% tissue culture infective dose ($TCID_{50}$) endpoint was 10^8 per ml.

Cell cultures. Primary mouse cells were obtained by trypsinization (0.05% in glucose-potassium-sodium solution) of the minced and rinsed fragments of 11-14 day old whole mouse fetuses for 45-60 minutes at room temperature. The cells were harvested from the supernatant fluid of the gravity-separated trypsinate and tissue fragments by centrifugation at 800 rpm for 10 minutes. The cell button was suspended in growth medium (GM), consisting of 10% calf serum and 90% E-BSS; the cells were enumerated in a hemocytometer, and seeded in 200 ml volume glass bottles at a concentration of 10^7 cells per 10 ml. Subsequently, mouse fetal fibroblastic cells were dispersed by trypsin (0.1%) and passed into 16 × 125 mm glass tubes at a concentration of 200,000 cells per 1.0 ml of GM. These replicate cultures of mouse fetal cells, nourished with MM, served as the culture system for the experiments to be described.

Viral RNA. Ten per cent tissue suspension of stock virus was diluted 2-fold in a mixture of dupanol (0.5%), bentonite (1.0 mg per ml) and Tris acetate buffer (0.01 M, pH 6.8). Freshly prepared buffer-saturated phenol was added in equal volume to the stock virus mixture, RNA extracted at 50°C for 5 minutes, and the preparation spun down at 8000 RPM for 5 minutes in a refrigerated centrifuge (5°C, Servall). The procedure was repeated once and then the supernatant fluid was extracted thrice at room temperature with an equal volume of buffer-saturated ether with subsequent removal of the ether layer and a flush with bubbled nitrogen for 15 minutes.

The RNA content of the phenol extracts of mengovirus and coxsackievirus A6 tissue suspensions, assayed by the orcinol method, was 55 and 48 μg per 0.5 ml respectively. The titer of mengovirus RNA in cultures of primary mouse fetal cells was 10^2 $TCID_{50}$ per ml.

Ribonuclease. Stock beef pancreatic ribonuclease‡ (RNAse), 1.0 mg per ml of distilled water and stored at —15°C, was employed to insure the biochemical validity of the RNA preparation. The RNAse was diluted in Tris acetate buffer to a final concentration of 10 μg per ml and incubated with viral RNA for 15 minutes at room temperature prior to inoculation of mouse fetal cell cultures. Otherwise viral RNA was incubated with equivalent volumes of buffer.

Inoculation of mouse cells with viral RNA. Cell sheets were rinsed once with 2.2 M $MgSO_4$ prior to addition of 0.1 ml volumes of viral RNA-buffer or viral RNA-RNAse mixtures to the cultures. The cells were exposed to these inocula for 15 minutes at room temperature followed by 3 rinses with BSS. Finally each culture was fed with 1.0 ml of MM and incubated at 37°C for 24-48 hours. At 24 and 48 hours the cultures were inspected for the presence of cytopathic change, then the cells and liquid medium were harvested by freezing and thawing thrice for subsequent assay of virus.

Inoculation of newborn mice with viral

‡ Worthington Biochemical Corp., Freehold, N. J.

TABLE I. Susceptibility of Mouse Fetal Cells to Mengovirus RNA.* Yield of newly synthesized virus.†

Time	Viral RNA	Viral RNA + RNAse
24 hr	$10^{2.5}$ $TCID_{50}$/0.1 ml	None
48 "	$10^{6.3}$ $TCID_{50}$/0.1 ml	"

* Extracted from weanling mouse brain seed pool, stock virus titer 10^8 $TCID_{50}$/ml, yielding 55 μg/0.5 ml of RNA.

† Assayed *in vitro* in tube cultures of mouse fetal cells; newly synthesized virus also lethal for newborn mice.

RNA. Viral RNA-buffer or viral RNA-RNAse mixtures were inoculated IC in 0.01 ml volumes into litters of 1-day old newborn mice. The mice were observed for subsequent hind limb paralysis and death.

Results. Susceptibility of mouse fetal cells to viral RNA. Mengovirus RNA successfully infected mouse fetal cells, producing characteristic cytopathic change. Freeze-thaw lysates of cultures harvested after 24 and 48 hours of incubation with mengovirus RNA yielded $10^{2.5}$ and $10^{6.3}$ $TCID_{50}$ per 0.1 ml of complete virus, respectively, by assay in mouse fetal cells (Table I). Lysates were also lethal for newborn mice. Mengovirus RNA pre-incubated with RNAse was non-infective for mouse fetal cells, as was viral RNA adsorbed to cells sheets in the absence of a prior rinse with hypertonic $MgSO_4$.

Excepting slight cell toxicity due to the brief exposure to hypertonic $MgSO_4$ during viral RNA adsorption, no cytopathic changes occurred in cultures infected with coxsackievirus A6 RNA. These experiments were repeated several times and on no occasion did coxsackievirus A6 RNA prove cytopathic for mouse fetal cells. However, if infective RNA would program only one cycle of new virus synthesis and new virions could not attach to receptorless cells it is conceivable that cytolysis would be subtle if noted at all. Thus, cultures incubated with coxsackievirus A6 RNA were harvested after 24 and 48 hours and the lysates assayed by IP inoculation of 1-4 day old newborn mice. With mouse-lethality as the endpoint there was no evidence for even a single cycle of complete virus synthesis in mouse fetal cells exposed to coxsackievirus A6 RNA.

Susceptibility of newborn mice to viral RNA. Undiluted mengovirus RNA produced 90% lethality in 24-hour old newborn mice within 6 days following IC inoculation. Mengovirus RNA pre-incubated with RNAse failed to cause hind limb paralysis and death in newborn mice.

On the other hand, IC inoculation of coxsackievirus A6 RNA failed to sicken or kill newborn mice.

Susceptibility of mouse fetal tissue suspensions to coxsackievirus A6. Nine-day old mouse fetuses were removed astptically, decapitated, and the torsos rinsed, minced with crossed scalpel blades, and rinsed again. The tissue fragments were centrifuged at 1000 rpm and suspended by volume in MM at a concentration of 5%. This procedure was performed without the use of trypsin or other proteolytic enzymes. Replicate suspensions of tissue were distributed in 1.0 ml volumes into 13 × 100 screw cap tubes, placed in a New Brunswick gyrotory shaker bath at 37°C, and infected with approximately 10^5 NM IP LD_{50} of coxsackievirus A6. Similar inocula of virus were added to tubes containing only 1.0 ml of MM as thermostability controls. The entire contents of tubes, experimental and control, were removed at zero time, 1, 3, 6, and 8 days, frozen and thawed thrice, the debris centrifuged at 1500 rpm and the supernatant fluid stored at −50°C for subsequent assay of virus. One- to 4-day old newborn mice were inoculated IP with 0.05 ml volumes of serial 10-fold dilutions of test fluids in MM and inspected daily for hind limb paralysis or lethality. These experiments revealed no evidence for the multiplication of coxsackievirus A6 in non-trypsinized 5% suspensions of mouse fetal tissue (Figure).

Coxsackievirus A6 inhibitors in mouse fetal tissue suspensions. To interpret further the previous experiment a mouse fetal tissue mince was prepared in similar fashion and incubated with $10^{6.6}$ NM IP LD_{50} of coxsackievirus A6 at 37°C for 1 hour. Controls consisted of similar infective inocula incubated with MM. The supernatant fluids from the experimental and control incubation mixtures were assayed in newborn mice as above. There was no evidence for neutralization of coxsackievirus A6 by mouse fetal tissue.

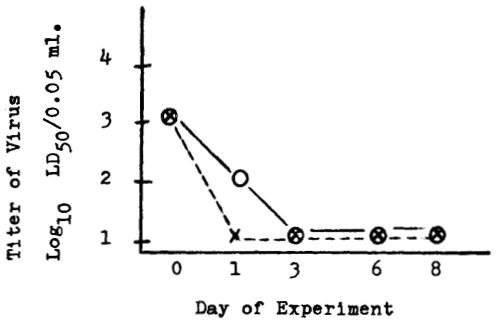

FIG. 1. Lack of multiplication of coxsackievirus A6 in 5% suspension of non-trypsinized mouse fetal tissue. O, tissue; X, medium control. Virus assay in 1-4 day old newborn mice; limit of virus detection, 10 NM IP LD_{50}.

Discussion. Several group A coxsackieviruses, while pathogenic for the intact mouse host, multiply inconsistently in mouse cells or tissue *in vitro* (4-8).

While multiplication of group A coxsackieviruses occurs in the skeletal muscle of the newborn mouse, cultivation of cells from whole fetal mouse tissue may effect deletion or dilution of potentially susceptible cells. However, mengovirus passed in mouse brain multiplied readily *in vitro* in cells derived from whole fetal mouse tissue.

Enterovirus receptors may be lost from otherwise susceptible mouse cells following dispersion by trypsin and cultivation *in vitro*. Zajac and Crowell(9) described the transient inactivation of some enteroviral receptors on HeLa cells following treatment with 1% solutioins of trypsin and chymotrypsin. McLaren, Holland and Syverton(10) demonstrated that sustained cultivation of cells *in vitro* may be associated with the loss of receptors. Infection of mouse cells *in vitro* with coxsackievirus A6 RNA should circumvent viral receptors and since it was unsuccessful, it suggests that either a faulty intracellular synthesizing mechanism or a defective viral genome is involved. This conclusion is supported by the fact that suspensions of whole mouse fetal tissue, untreated with possible receptor-destroying proteolytic enzymes, failed to support multiplication of this virus. The conjecture that proteinaceous non-specific viral inhibitors might have obscured the potential significance of these experiments was dismissed by the failure of tissue suspensions to neutralize virus.

Cultivation of mouse fetal tissue *in vitro* may alter the competency of the host cell to translate the genetic information of certain types of infectious RNA into synthesis of viral subunits or their assembly into complete virions. Why coxsackievirus A6 RNA should fail where mengovirus RNA succeeds is puzzling. In spite of the quantitative biochemical congruity between the two phenol-extracted preparations of RNA, incompetency may be that of the infectious RNA, either qualitatively the coxsackievirus A6 nucleoprotein core itself or that extracted in the laboratory. The fact that coxsackievirus A6 RNA failed to induce illness or death in newborn mice, ordinarily very susceptible to complete virus, supports this contention. Simultaneously, mengovirus RNA produced hind limb paralysis and death in newborn mice, just as it had evoked cytopathogenic multiplication of complete mengovirus in mouse fetal cells *in vitro*.

The perplexing paradox of the insusceptibility of mouse cells *in vitro* appears to circumscribe either a subtle alteration of the virus-synthesizing mechanism of the cultivated mouse fetal cells, specific for coxsackievirus A6, or the viral genome itself.

Summary. The paradoxical insusceptibility of mouse cells *in vitro* to mouse-lethal coxsackievirus A6 was investigated further by infection of mouse fetal cells and newborn mice with RNA extracted from coxsackievirus A6 and mengovirus. Mengovirus RNA induced viral replication *in vitro* and lethality *in vivo*, while coxsackie A6 RNA failed to do either. Intact coxsackievirus A6 was also incapable of multiplication in non-trypsinized suspensions of mouse fetal tissue. These studies suggest that loss of viral receptor sites on mouse fetal cells, subsequent to dispersion by trypsin and cultivation, does not explain the insusceptibility of mouse cells *in vitro* to coxsackievirus A6. The insusceptibility of mouse cells *in vitro* may represent either disruption of the virus-synthesizing potential of cultivated cells, rather specific in nature, or the biologic integrity of the viral genome.

The authors would like to express their sincere gratitude to Dr. Leroy C. McLaren for advice concerning the initial scope of this work, to Dr. Ernest D. Gray and Mr. Joseph Ferretti for consultation regarding biochemical methods, and to Dr. David T. Imagawa for review of the manuscript. Miss Ann Barna provided stenographic assistance in the preparation of the manuscript.

1. St. Geme, J. W., Jr., J. Bacteriol., 1964, v87, 969.
2. Came, P. E., Crowell, R. L., Virology, 1964, v23, 542.
3. Dick, G. W. A., Best, A. M., Haddow, A. J., Smithburn, K. C., Lancet, 1948, v2, 286.
4. Slater, E. A., Syverton, J. T., Proc. Soc. Exp. Biol. Med., 1950, v74, 509.
5. Stulberg, C. S., Schapira, R., Eidam, C. R., J. Immunol., 1954, v72, 107.
6. Fujita, T., Sugai, T., and Okuno, V., Biken's J., 1959, v2, 295.
7. Lenahan, M. F., Wenner, H. A., Proc. Soc. Exp. Biol. Med., 1961, v107, 544.
8. Moore, M. L., Hooser, L. E., Davis, E. V., Siem, R. A., J. Infect. Dis., 1964, v114, 189.
9. Zajac, I., Crowell, R. L., J. Bact., 1965, v89, 1097.
10. McLaren, L. C., Holland, J. J., Syverton, J. T., J. Exp. Med., 1960, v112, 581.

Received December 5, 1966. P.S.E.B.M., 1967, v125.

Adenosine Triphosphate in the *in vitro* Hemolytic Tests for Paroxysmal Nocturnal Hemoglobinuria.* (32013)

FARID I. HAURANI, RAYMOND J. VIVACQUA, AND ALLAN J. ERSLEV
Cardeza Foundation for Hematologic Research, Jefferson Medical College, Philadelphia, Pa.

During the performance of the autohemolysis test on the blood of a patient with paroxysmal nocturnal hemoglobinuria (PNH), it was observed that adenosine triphosphate (ATP) was effective in inhibiting the hemolysis. When this finding was extended to the acid-thrombin test of PNH red blood cells, a similar inhibition of hemolysis was observed. Further studies which form the basis of this report, suggest that this inhibition is caused by the effect of ATP as a chelating agent of magnesium (a necessary component of the PNH *in vitro* hemolytic system) rather than by being a source of intracellular energy or by virtue of its effect on the pH of the system.

Methods. The in vitro *hemolytic system.* The *in vitro* system(1) utilized for the acid test in these studies consisted of 0.5 ml normal serum, 0.05 ml 0.2 N HCl, additives in a volume of 0.05 ml and 0.05 ml of PNH (or normal) red blood cells suspended in saline with a hematocrit of approximately 50%. The pH was determined before addition of the red blood cells. Incubation was carried out in a water bath for 15 minutes and the blood specimens were centrifuged for 2 minutes at 1000 rpm. Hemoglobin content was determined in the supernatant by the benzidine or cyanmethemoglobin method (1). In the acid-thrombin test, thrombin 0.05 ml (approximately 50 NIH units) was added before the red blood cells.

Preparation of Mg ATP. Mg ATP was prepared from barium ATP using excess of magnesium sulfate(2). The Mg, adenine, free phosphorus and total phosphorus were determined. The molarity of the Mg ATP solution prepared on 2 separate occasions was approximately 0.20 M/l, as determined by the adenine(3) and total phosphorus content(4). The molarity of Mg in the Mg ATP solution was 0.36 M/l and 0.38 M/l on 2 separate occasions (Mg was determined by A. L. Chaney Chemical Laboratory, Glendale, Calif.). Since the expected molar binding of ATP to Mg is in the ratio of 1:1(5), there was 0.16 and 0.18 M/l of excess magnesium. The free phosphorus(4) of the Mg ATP was less than 1%.

Phosphorolysis of ATP. Although it was not likely that phosphorolysis of ATP by serum phosphatase with release of energy and ADP occurred within the short time

* Supported by USPHS Grant CA 05554-05 and Clinical Research Center Grant FR-72.