TABLE VI. Growth Retardation of HAD #1 in Embryonated Egg by Hadacidin and Other Antitumor Compounds.

Compound	Dose, mg/egg	Embryo mortality	Growth retardation, % Embryo	Tumor
Hadacidin—Monosodium salt	6.0	3/24*	3.5 (0– 7)†	61 (56– 65)†
	7.5	4/21*	20 (18–22)†	68 (62– 73)†
Monopotassium salt	6.0	0/6	14	59
Monoammonium salt	5.0	1/6	27	52
Methyl ester	7.5	0/6	14	57
Triethylene melamine	.025	2/48‡	10 (0–23)†	74 (48–100)†
Azaserine	2.0	5/9	41	75
	1.0	2/9	20	62
6-Mercaptopurine	8.0	3/9	6	63
	4.0	0/9	3	27

* Results derived from 2 different experiments.
† No. in parentheses refer to range of values observed.
‡ Results derived from 8 different experiments; 6 eggs/treatment.

Our results with HAD #1 and HEP #3 have been confirmed by Harris and Yap-Guevera who found hadacidin effective also against A-42 and HS #1(8). We have confirmed their observations with A-42. In our hands the strain of HEP #3 tumor now in use proved the least sensitive of the tumors studied. However, an earlier employed strain of HEP #3 was as sensitive to hadacidin as HAD #1.

Summary. A new crystalline antitumor agent, hadacidin, has been discovered in fermentation broths of *Penicillium frequentans* Westling. The human tumor-embryonated egg system was used to guide fermentation and isolation studies. Against HAD #1 the tumor ID_{50} of hadacidin is about 3 mg per egg. Hadacidin is active also against HEP #3 and A-42 in the embryonated egg. On a weight basis, hadacidin has about the same range of activity as azaserine and 6-mercaptopurine but has only one per cent the activity of the alkylating agent, triethylene melamine.

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Observations on the Properties and Prevalence of Coe Virus.* (27357)

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During viral isolation studies on material obtained from the Bangkok (Thailand) cholera outbreak in 1958, an agent was isolated (1) which bore some resemblance to the Coe virus. In comparing the 2 agents, which

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proved to be unrelated, certain observations were made on the biological characteristics of the Coe virus and on the prevalence of specific antibody in certain population groups. These observations form the basis of this report. The Coe virus was originally isolated from cases of acute respiratory disease in California(2). Even though this virus does not grow in monkey kidney cell cultures, it produces enterovirus-like CPE in human cells (HeLa, Kb, FL), shows low grade pathogenicity for newborn swiss albino mice and is ether resistant. The virus isolated by the Pereiras in England(3) and shown to be identical with the Coe virus was demonstrated by filtration experiment through graded gradochol membranes to have a particle size of about 20 mμ, while Frommhagen's electron microscopic measurements on the original Coe virus indicated somewhat larger size(4).

Materials and general methods. Virus. The Coe virus, kindly supplied by Dr. E. H. Lennette as sixth HeLa passage TC fluid, was propagated in human amnion cell cultures, FL line(6). The virus multiplied as satisfactorily in these cells as in the HeLa cells.

Virus assay. Serial 1 $\log_{10}$ dilutions of infected TC fluid were inoculated into 4 FL monolayer cell culture tubes in 0.1 ml volume. The $TCID_{50}$ was determined by the method of Reed and Muench(7). For plaque assay, the same volume was inoculated on FL monolayer cell cultures.

Tissue culture. FL cells were maintained in 32 oz. bottles by weekly passage. Hanks' BSS with 0.5% lactalbumin hydrolysate, + 10% bovine serum was used as growth medium. TC culture tubes were prepared by inoculating 25,000 cells per tube in 1 ml growth medium. Tubes were ready for use on the 4-5th day. M199 – BAF + 1% bovine serum was used as maintenance medium. The same medium was used for preparing virus pools. For plaque assay, 1,500,000 cells were inoculated into 3 oz. bottles containing 10 ml of growth medium which was renewed on the 3rd day. The cultures were ready for use on the 5-6th day. After introduction of the virus, the bottles were kept at 37°C for 30 min. The agar overlay, 7.5 ml per bottle, consisted of Eagle's medium containing 1.5% Noble Agar, to which 10% of bovine serum and 10% tryptose phosphate broth were added. The small (2-5 mm), uniform, clear plaques began to appear on the 3rd day, and were counted on the 7-9th day.

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(14).

Electron microscopy. The virus pool, approximately 1,000 ml—titer: $10^{6.5}$ $TCID_{50}$/ml—was purified by Genetron treatment(8), then concentrated by dialyzation against 50% poly-vinyl-pyrollidon and, after another cycle of Genetron treatment, was sedimented in the ultracentrifuge at 40,000 rpm for 3 hr (Spinco Model L), and the pellet was taken up in 0.9% salt solution. Kellenberger's(9) agar method was employed for mounting the virus concentrate on the collodium membrane. RCA Model EMU 3F electron microscope was used.

Experimental results. Heat inactivation. At 45 and 56°C the heat inactivation rate was similar to those of human enteroviruses (Fig. 1) in agreement with the data of Jordan(11).

Adsorption to FL monolayer cells. Adsorption of virus was measured by inoculating FL monolayer cell cultures in triplicate with approximately 100 PFU virus in 0.2 ml volume at 37°C, pH 7.4. At regular intervals thereafter the inoculum was washed out, and the cells were overlaid with agar medium. The 180 min plaque count was taken as 100% (Fig. 2); 50% of the virus was adsorbed to the cells in a short time (15 min) after which the rate of adsorption diminished.

Multiplication of Coe virus. One step growth curve experiment (Fig. 3) was done using FL cells in tubes, in serum-free M199-BAF medium at 37°C. A large viral inoculum (0.4 ml) of high titered virus was used to achieve high multiplicity of infection of cells. After incubation at 37°C for 90 minutes the fluid was decanted and each tube rinsed 3 times with 5 ml of Hanks' solution. Each tube then received 1 ml of M199-BAF before being returned to the 37°C incubator.

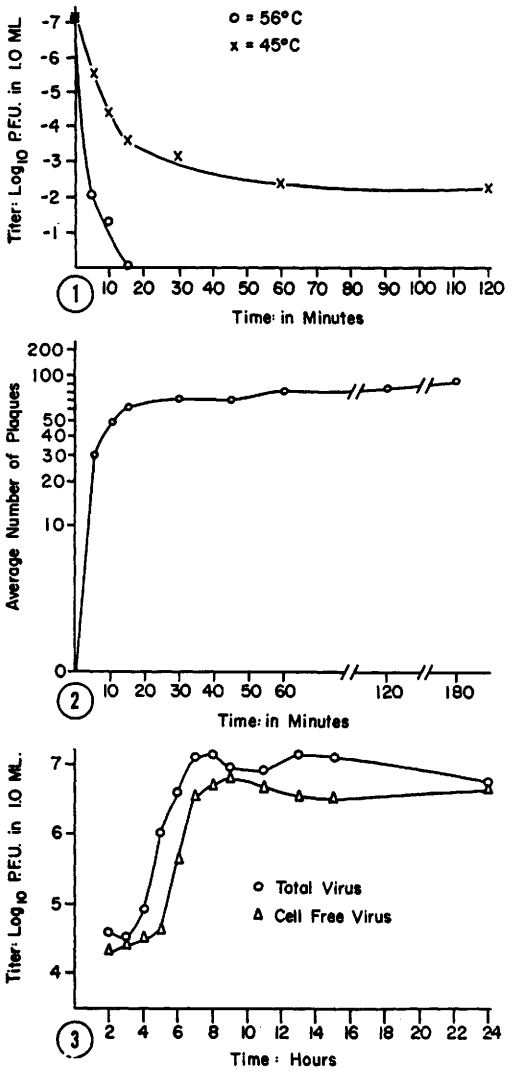

FIG. 1. Heat inactivation of Coe virus.
FIG. 2. Adsorption of Coe virus to FL cells.
FIG. 3. One step growth curve of Coe virus in FL cells. Multiplicity of infection: 16.

At intervals 5 tubes were withdrawn and virus content of the supernatant and of total culture was determined. The intracellular infectious virus appeared after 3 hr of eclipse period (Fig. 3) and increased exponentially until the seventh hour. The virus began to appear in the supernatant approximately 4½ hours after inoculation, and showed a similar growth curve. The virus was rapidly released from the cells into the medium. The virus yield per cell in this experiment was about 100 PFU per cells. This is in the lower range found with enteroviruses in monkey kidney cell cultures.

Nature of the virus. Indirect evidence(10, 12, 13) indicates that the virus is an RNA virus. 10 μg/ml 5 bromo-deoxy-uridine, or 5 Fluoro-deoxy-uridine, (10^{-6} M) aminopterin 10 mg/ml did not inhibit multiplication of Coe virus in 8-hour pretreated FL cells. Both compounds in concentrations used inhibited multiplication of vaccinia virus, and were reversed by addition of thymidine (3×10^5 M). Higher concentrations of these inhibitors, up to 100-fold, failed to interfere with multiplication of Coe virus.

Morphology of the virus. In platinum shadowed, air dried preparation the virus appeared as a uniform, round particle in the size range of human enteroviruses. The average diameter of the free virus particles was found to be 39.4 mμ $\pm$ 1.7 mμ (Fig. 4).

Serological identity of the virus. Specific, high titered (50% neutralization end point, 1:7,500), immune-serum was prepared in a rhesus monkey, using concentrated, purified virus as antigen. By cross neutralization test the identity of the Coe virus with Coxsackie A-21 was confirmed(15). The virus was found to be serologically unrelated to the following recently described viruses which produce no CPE in monkey kidney cell cultures:

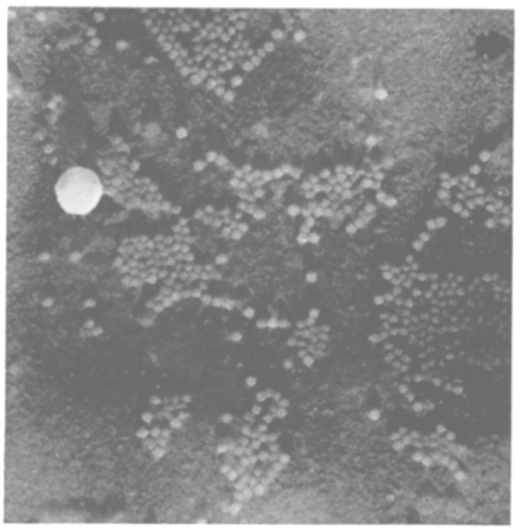

FIG. 4. Electron micrograph of an air-dried preparation of Coe virus. Magnification $\times$ 32,160; shadowed with platinum-palladium.

Pett(16), HSO(17), and 4 viruses described by us(18).

Prevalence of neutralizing antibodies in the normal population. The presence of antibodies in sera drawn in 1959-1961 from several Pittsburgh population groups was determined by neutralization tests. For screening, a 1:10 dilution of inactivated human serum was used against 100 $TCID_{50}$ virus. In general, the results paralleled those reported by the Pereiras in Great Britain(3). Of the 98 sera from children tested, 2 (2%) had significant antibody titers. Among young adults, (20-30 yr. age group) antibodies were found in 6 out of 116 individuals (5.2%). Middle-aged adults showed a higher incidence of antibodies: 26 out of 215 (12.1%) being positive. Sera obtained in 1959 showed approximately the same percentage of positives as sera obtained in 1960 or in the Spring of 1961. The antibody titer showed a range from 1:8 to 1:128; the most commonly encountered titer was 1:16. These data indicate the occurrence of Coe virus infections in the Pittsburgh area.

A small group of sera from Thailand was also tested. No significant antibody titers were found and only low grade, presumably non-specific, inhibition was encountered. However, 10 of the 39 sera from Japan showed the presence of antibodies in significant titer (1:8-1:128).

Discussion. The importance of the Coe virus isolated by Lennette(2), as a human pathogen, is confirmed by the study of Johnston *et al.*(19) on the extensive outbreak of acute respiratory illness at Camp Lejeune, N. C. Isolation of the virus was reported from Great Britain by the Pereiras(3) and from Holland by Van der Veen(20). Recent information indicates the close relationship of this virus to the human enteroviruses(2,3,4,5,11). Data on heat inactivation and on the biological characteristics of the Coe virus (adsorption, multiplication) confirm this relationship. The indirect evidence for this being an RNA virus is established by Frommhagen's direct determination of RNA content of the virus. His sedimentation data in caesium chloride density gradient, and ultraviolet adsorption measurements are similar to those of Pett and human enteroviruses(4). His measurement of particle size in air dried preparations by the electron microscope gives larger average diameter, 43 $\pm$ 3 mμ, than our data of 39.4 $\pm$ 1.7 mμ. The presence of specific antibody levels in a significant number of sera drawn from several population groups in the Pittsburgh area suggests that this agent has been prevalent in this locality in the past.

Summary. Determination of some biological characteristics of Coe virus indicates the close resemblance of this agent to the human enterovirus group. The presence of antibodies in sera of normal human population in the Pittsburgh area suggests a past exposure to the Coe virus.

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Effect of Chromium, Cadmium and Lead on Serum Cholesterol of Rats. (27358)

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Curran showed that chromium compounds considerably enhanced hepatic synthesis of cholesterol and fatty acids by rat liver, both *in vitro* and *in vivo*(1). Therefore, it appeared rewarding to determine what effect small amounts of chromium and 2 accumulating metals, cadmium and lead, given over the long term, might have on circulating levels of total cholesterol in rats on a regime rigidly restricted as to contact with these trace metals.

Method. Eighty weanling male rats of the Long-Evans strain were bred in quarters remotely situated on a hilltop and especially constructed so as to avoid environmental contact with these and other metals. The diet was composed of whole seed rye flour (60%), powdered skim milk (30%), corn oil (9%) and sodium chloride (1%), with added vitamins and ferrous sulfate (0.01%)(2). Food contained no detectable cadmium(3), 0.2 μg/g of lead(4), and 0.1 μg/g of chromium (5). Cages were made of acrylic resin, covers of stainless steel, water bottles of polyethylene. Laboratory air from near a forest was filtered through electrostatic precipitators to avoid airborne lead and cadmium, found in snow contaminated by automobile exhausts(3,4). Environmental temperature approximated 24°C. Food and water were repeatedly analyzed for trace metals by microanalytical chemical methods(3,4,5).

Soft water from a forest spring was passed through a polyresin water softener and through both a bulk and a laboratory deionizer and contained no detectable metal. To it was added 5 essential trace metals in amounts approximating those contained in commercial laboratory diets: Zinc 50 ppm., copper 10 ppm., manganese 10 ppm., cobalt 1 ppm., and molybdenum 1 ppm. as the chlorides, acetates or molybdates. To each group of 20 rats, 4 to a cage, was given 5 ppm. of metal as lead acetate, cadmium chloride or chromic acetate in their drinking water, one group of 20 serving as control. Animals remained on this regimen for 10 months or longer and were subjected to no controllable stresses, all being handled alike.

Blood was obtained from the tails of pairs of animals and serum cholesterol measured by the method of Abell *et al.*(6) in duplicate. Hepatic cholesterol was measured by the same method after acetone-alcohol extraction in male animals aged 307 to 372 days dying spontaneously.

Results. Mean serum cholesterol levels are shown in Table I. Significant alterations from the controls ($p<0.01$) occurred in those given lead and cadmium, but in a direction opposite to what might be expected, *i.e.*, by depression. The level for the lead-fed animals was significantly lower than that of the animals given chromium ($p \pm 0.01$), but did not differ statistically from that of the cadmium group. Nor did the cadmium and chromium groups so differ.

Analyses of livers for metals by microanalytic chemical methods(3,4,5) revealed no detectable cadmium in the controls or lead-fed groups, while in those taking cadmium concentrations approximated 1.7 μg/g wet tissue. Lead concentrations were 0-0.33 μg/g in the control, 0-0.49 μg/g in the cadmium-fed and 2.0-3.1 μg/g in the lead-fed

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