

LASTING BIOLOGICAL EFFECTS OF EARLY ENVIRONMENTAL
INFLUENCES*

IV. NOTES ON THE PHYSICOCHEMICAL AND IMMUNOLOGICAL
CHARACTERISTICS OF AN ENTEROVIRUS THAT DEPRESSES
THE GROWTH OF MICE

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A lasting depression of body weight can be achieved consistently by contaminating newborn mice, specific pathogen-free (SPF), with a filterable agent derived from the intestine of mice raised under ordinary conditions of husbandry (1, 2). Adult SPF mice, contaminated with this agent after birth, exhibit a variety of metabolic abnormalities, such as reduced ability to incorporate amino acids and to utilize dietary nitrogen (2).

Although the active filterable agent has been cultivated in tissue culture (1), there is as yet no convincing evidence that it has been completely separated from other agents.

For the sake of convenience, it will be tentatively designated here as enterovirus, using as single criterion of its pathogenicity its ability to cause a lasting depression of body weight of SPF mice. The present report describes the effect of certain physical, chemical, and immunological procedures on the biological activities of preparations of this agent administered by the oral route into newborn mice.

Materials and Methods

Experimental Animals and Infective Material.—All experiments were carried out with pathogen-free mice of the COBS strain (Caesarian-obtained, barrier-sustained, obtained from Charles River Breeding Laboratories, Inc., North Wilmington, Mass.). The origin and preparation of the filterable agent have been described in an earlier publication (2). In brief, the intestines of 1 wk old COBS mice which had been contaminated with the enterovirus were homogenized with a Teflon grinder; the homogenate diluted in Tris-buffered salt solution was then passed through a Millipore filter of 0.45 μ porosity. This filtrate was administered *per os* to 2-day old COBS mice, which were from then on maintained with their dams without further disturbance. Water and D&G pellets (Dietrich and Gambrell, Inc., Frederick, Md.) were given ad libitum as described earlier (2).

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Titration of Virus Infectivity.—Virus infectivity was titrated in newborn mice by the 50% end point of body weight depression (ID_{50} titer) according to the Reed and Muench method (3, 4). Body weight depression was expressed as the mean of depression ± 1.6 standard deviation, which included 95% population of affected mice. The tests were carried out with serial 10-fold dilutions of filtrate. The body weights were determined 5 days after infection. In groups of 30 mice weighed at 1 wk of age, the limits of body weight depression ranged from 2.6 to 4.6 g in males and 2.3 to 4.3 g in females.

Physicochemical Characterization of Virus.—The intestinal filtrate was treated by various physical and chemical procedures within a few hr after filtration. Comparative determinations of infectivity were made before and after each treatment.

Ultracentrifugation: The intestinal filtrate was centrifuged in a Spinco Model L-2 Ultracentrifuge using Rotor No. 40 at 39,000 rpm for $2\frac{1}{2}$ hr. The sediments were resuspended in a volume of diluent equal to that of the original intestinal filtrate. Both supernatant fluids and resuspended sediments were titrated for infectivity.

Ultraviolet irradiation: A volume of 1.0 ml of filtrate was placed in a Petri dish, 5 cm in diameter, and irradiated for 10 min at 4°C with an ultraviolet lamp (G15T8 General Sylvania), placed at 15 cm distance from the sample.

pH 4.3 precipitation: The filtrate at pH 7.8 was adjusted to pH 4.3 by addition of 0.1 N hydrochloric acid with constant stirring, and the acidified material was allowed to stand at this reaction for 24 hr at 4°C. After centrifugation, the sediment was resuspended in Tris-buffered salt solution at pH 7.8 and then readjusted to the original volume.

Precipitation in 50% ammonium sulfate solution: Ammonium sulfate was added to the filtrate up to a final concentration of 50%. The solution was stored at 4°C overnight, then dialyzed in a cellophane tube against distilled water until no sulfate ion could be detected in the dialysate.

Heating at 56°C: The filtrate at pH 7.8 was heated in a water bath at 56°C for 1 hr.

Ether treatment: The filtrate was treated twice with an equal volume of ether and the mixture was allowed to stand overnight at 4°C. The upper ether layer was removed with a pipette.

Dialysis: The filtrate was dialyzed in a cellophane tube against distilled water at 4°C for 24 hr and the dialyzed residue was adjusted to a constant volume.

Treatment with trypsin: The filtrate was treated with 0.4% trypsin solution for 30 min at 40°C, pH 7.8 (bovine pancreas trypsin, 2 × crystallized, 10,000 units activity/mg, obtained from Sigma Chemical Co., St. Louis, Mo.).

Treatment with nucleases: 5 ml of filtrate was treated with 1.0 ml of 100 mcg/ml deoxyribonuclease or 1.0 ml of 100 mcg/ml ribonuclease. The mixtures were incubated at 37°C pH 7.8 for 20 min. (The crystalline enzymes were obtained from Worthington Biochemical Corp., Freehold, N. J.)

Immunological Properties.—

Hemagglutination test: The hemagglutinating activities of intestinal filtrates prepared from infected and noninfected mice were titrated by adding 0.1 ml of serial two-fold dilutions to 0.1 ml of 1% suspension of mouse blood cells and incubating the mixtures at 4°C for 1 hr. Results were expressed as the reciprocals of the final dilutions producing hemagglutination.

Neutralizing antibody: Rabbits, $1\frac{1}{2}$ yr of age, 1.5–2 kg body weight, received by the intravenous route five successive doses at 5-day intervals of 1.0 ml intestinal filtrates prepared from infected or noninfected mice. 1 month after the fifth injection, the rabbits received two other 1.0 ml doses of filtrates at 5-day intervals. 1 wk after the last injection, 10–15 ml of blood was withdrawn from each rabbit and the serum separated. Serial five-fold dilutions of serum were mixed with equal volumes of 1:10 dilution of intestinal filtrate and the mix-

tures incubated at 37°C for 45 min. The infectivity of incubated mixtures was titrated in newborn mice as described above.

Ultracentrifugation of Infected Filtrate in Sucrose Density Gradients.—A cellulose nitrate tube (0.5 × 2 inches) received 2.25 ml of 50% and 2.25 ml 20% (w/v) sucrose in Tris-buffered salt solution through mixing gradient chambers. A volume of 0.5 ml of intestinal filtrate, concentrated five times, was floated on the top of the sugar gradient solution. The other tubes were each filled with 2.5 ml of 50% and 20% sucrose solution. The tubes were centrifuged at 4°C in a Spinco swinging bucket, SW 39, at 39,000 rpm for 2 hr. The centrifuge tube was removed and the bottom of the tube punctured with a 26½ gauge hypodermic needle;

TABLE I
Effect of Various Physicochemical Treatments on Infectivity of Intestinal Filtrates Prepared from COBS Mice Infected Neonatally

Treatment	Infectivity*	Infective Units‡	Specific Infectivity§
Control	10 ^{-4.33}	2.40	4.22
Ultracentrifugation			
Supernatant fluid	No activity	—	—
Sediment	10 ^{-4.15}	1.41	3.02
50% ammonium sulfate			
Sediment	10 ^{-3.56}	0.364	0.714
pH 4.3 precipitation			
Sediment	10 ^{-2.11}	0.013	0.033
Dialyzed residue	10 ^{-6.41}	251	552
Ultraviolet irradiation	10 ^{-0.34}	0.0002	0.0004
Heating at 56°C	10 ^{-5.00}	10.0	18.5
Ether	10 ^{-6.25}	178	313
Trypsin	10 ^{-4.50}	3.16	4.30
Deoxyribonuclease	10 ^{-6.27}	186	310
Ribonuclease	10 ^{-6.33}	213	375

* ID₅₀.

‡ 1:ID₅₀ × 10⁴.

§ Infective Units/mg. Protein × 10³.

10 drops of sample solution were collected from the tube. Infectivity, hemagglutination, and protein content were measured for all fractions.

Murine Virus Antibody Determinations.—The procedures used for the serological tests are indicated in Table IV. The tests included hemagglutination-inhibition for antibodies against some of the most common mouse viruses (S): pneumonia virus of mice (PVM), reovirus type 3, Theiler's mouse encephalomyelitis (GD VII), K, polyoma, and Sendai virus; complement-fixation for antibodies against mouse adenovirus and mouse hepatitis.

RESULTS

Physicochemical Characteristics of Virus.—As seen in Table I, there was no activity in the supernatant fluid after ultracentrifugation of the intestinal filtrate; however, activity was recovered in the sediment. Most infectivity was lost after ultraviolet irradiation.

Precipitation with 50% ammonium sulfate resulted in a great loss of activity, but some of it was recovered in the precipitate.

Infectivity was almost completely destroyed by exposure at pH 4.3, but not by treatment with trypsin, ribonuclease, and deoxyribonuclease or by heating at 56°C.

TABLE II
Immunological Activity of Intestinal Filtrate

		Titer
Hemagglutinating titer of intestinal filtrate prepared from	Control mice	10
	Infected mice	80
Neutralizing titer of rabbit serum after immunization with intestinal filtrate prepared from	Control mice	0
	Infected mice	5

TABLE III
Distribution of Infectivity, Hemagglutinating Activity and Protein Content in Intestinal Filtrate of Infected Mice After Sucrose Gradient Ultracentrifugation

	Before centrifugation	Fractions after centrifugation								Total
		1	2	3	4	5	6	7	8	
Infectivity ID_{50}	$10^{-4.65}$	$10^{-2.00}$	$10^{-3.17}$	$10^{-2.87}$	$10^{-1.44}$	$10^{-3.25}$	$10^{-2.24}$	$10^{-5.50}$	$10^{-4.88}$	
Total infective units vol. $\times 1/ID_{50} \times 10^4$	22.4	0.006	0.093	0.046	0.002	0.111	0.011	19.8	1.50	
Infectivity recovered (%)	100	0.03	0.41	0.21	0.01	0.50	0.05	88.5	6.7	96.4
Total protein, mg	14.50	2.00	2.00	2.00	1.75	1.50	1.75	1.75	1.50	14.25
Infective units/mg protein $\times 10^4$	1.54	0.008	0.046	0.023	0.001	0.072	0.001	11.3	1.00	
Hemagglutination										
Titer	80	0	5	0	0	10	10	160	120	
Recovered, %	100	0	0.78	0	0	1.56	1.56	25.0	18.75	47.7
Per mg protein	27.6	0	1.56	0	0	4.16	3.57	91.4	70.6	

The fact that infectivity was greatly increased by dialysis and by treatment with ether, ribonuclease, or deoxyribonuclease, suggests that these procedures eliminated or destroyed some inhibitory substances.

Immunological Properties.—As seen in Table II, the intestinal filtrate of noninfected COBS mice showed hemagglutinating activity at dilution 1:10. However, the hemagglutinating titer of the filtrate of infected mice was much higher and reached the level of 80. Hemagglutination was observed with mouse blood cells, but not with the cells of sheep and guinea pig.

The serum of rabbits immunized with intestinal filtrate from infected mice

had a neutralizing titer of 5, whereas the serum of rabbits immunized with filtrate from normal mice was inactive in this regard.

Distributions of Infectivity, Hemagglutinating Activity and Protein Content in Intestinal Filtrate after Sucrose Gradient Ultracentrifugation.—As seen in Table

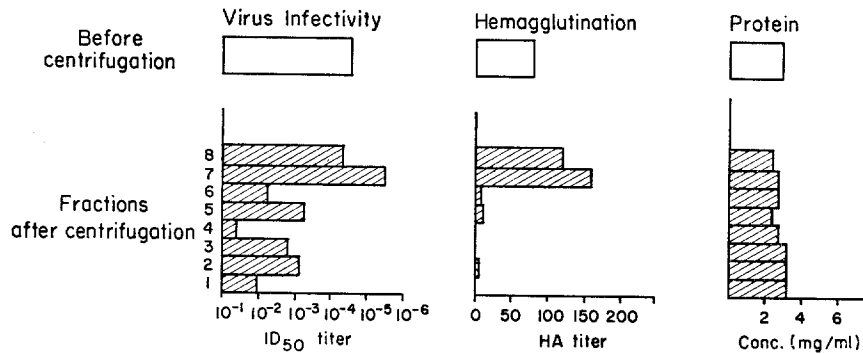


FIG. 1. Distribution of infectivity, hemagglutinating activity, and protein content in infected filtrate after sucrose gradient ultracentrifugation.

TABLE IV
*Antibody Determination in Serum of COBS Mice**

	Control mice	Infected mice
Hemagglutination inhibition		
PVM	Negative	Negative
Reovirus	"	"
Theiler's mouse encephalomyelitis (GD VII)	"	"
K	"	"
Polyoma	"	"
Sendai	"	"
Complement fixation		
Mouse adenovirus	"	"
Mouse hepatitis	9 Negative 3 Positive at 1:10	7 Negative 4 Positive at 1:10-1:20

* The serological tests were performed by Microbiological Associates, Inc. (Bethesda, Md.), on sera obtained from mice 8 to 9 wk of age. 11 mice were infected 2 days after birth, 12 were controls. The initial test dilutions of the sera were 1:10 for the viruses of mouse hepatitis, mouse adenovirus, K, and Sendai, and 1:20 for the other viruses.

III and Fig. 1, 88.5% of the total infectivity was recovered in fraction 7; some infectivity was also present in fraction 8. The total recovery of infectivity after centrifugation was 96.4%. The total recovery of protein content in the various fractions was 98.4%. The specific infectivity of fraction 7 was 7.4 times greater than that of the filtrate before centrifugation. Hemagglutinating activity was

not detected in fractions 1, 3, and 4, but there was some in fractions 2, 5, and 6. Interestingly enough, the highest levels of hemagglutinating activity were also present in fraction 7 (titer of 160) and in fraction 8 (titer of 120). The total recovery of the hemagglutinating activity after centrifugation was 47.7%. The specific hemagglutinating activity in fraction 7 was 3.3 times and in fraction 8, 2.6 times greater than in the nontreated filtrate.

Although infectivity was greatly increased after treatments by dialysis, ether, deoxyribonuclease or ribonuclease used separately, the combined effects of these various procedures did not result in any further increase of activity. In fact, both infectivity and protein content decreased rapidly in consecutive treatments, and the specific activity was completely lost in the final steps of attempts at purification.

Antibody Determination.—As seen in Table IV, the serum of adult mice, either infected or noninfected, exhibited no significant immunological activity against the following viruses: PVM, reovirus type 3, Theiler's encephalomyelitis (GDVII), K, polyoma, Sendai virus, mouse adenovirus, and mouse hepatitis.

SUMMARY

Physicochemical and immunological techniques have been used in an attempt to characterize a filterable agent, separated from the intestines of mice raised under ordinary conditions of husbandry, which produces a lasting depression of weight in specific pathogen-free (SPF) mice when administered to them orally shortly after birth. Although this agent has not yet been identified, it will be tentatively designated here as enterovirus.

The mouse enterovirus can be readily sedimented by ultracentrifugation and by precipitation at pH 4.3; it does not pass through cellophane membranes. Its infective power is completely destroyed by ultraviolet radiation, but is resistant to heating at 56°C, exposure to ether, treatment with trypsin, ribonuclease, and deoxyribonuclease.

Dialysis and treatment with ether and nucleases greatly increase the infective activity of the intestinal filtrates containing the enterovirus, a finding which suggests that these procedures eliminate or destroy some inhibitory substance(s).

The mouse enterovirus causes hemagglutination of mouse red blood cells. When injected into rabbits, it elicits in them an immune response that renders their serum capable of neutralizing its weight-depressing activity. As measured by inhibition of hemagglutination or complement fixation, the sera of infected mice do not exhibit any significant activity against usual mouse viruses.

Centrifugation of the mouse enterovirus in 50%–20% sucrose gradient gave almost complete recovery of the infectivity and of hemagglutinating activity in the same fraction. In contrast, the protein content of the material was distrib-

uted through the various fractions. Consequently, this procedure resulted in a marked increase of specific activity.

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