Progesterone dissolved in propylene glycol and administered intravenously to a pregnant woman also had a short life as ether extractable steroid. This procedure was followed by a marked rise in urinary sodium pregnandiol glucoronidate excretion.

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Recovery of the Coxsackie Group of Viruses from Human Sources. (17708)

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In 1948 this laboratory reported(1) on the presence of neutralizing antibodies in human sera against the virus of Newcastle Disease (NDV). NDV was not isolated from any of the material examined, and it was shown later (2,3) that the positive neutralization tests were due to a nonspecific heat-labile factor in the sera. Since frozen specimens from the cases described in the previous paper were still available, following the report by Dalldorf and Sickles(4) on the recovery of the Coxsackie virus from human feces(5), this material was re-examined by inoculation into suckling mice. The Coxsackie virus was obtained from both the feces and nasopharyngeal washings of many of these patients. Subsequently, more specimens were examined from various sources, and virus strains have been recovered in material from 97 individuals in 9 different states: Alabama, Colorado, Delaware, Florida, Georgia, Louisiana, Oklahoma, South Dakota and Tennessee. This report presents the results thus far obtained in the study of the viruses isolated.

Methods. (a) *Treatment of feces or oral washings.* Fecal and oral specimens were prepared according to the method of Howitt and Barnett(6) for the recovery of poliomyelitis virus. Twenty percent suspensions of feces were made in distilled water. These were spun in an angle centrifuge at 13,000 r.p.m. for 30 minutes in the cold room. One thousand units of penicillin and 10 mg of streptomycin were added for each ml of the supernatant fluid, and the mixtures were left at room temperature for 30 minutes before inoculation of 3-to-5-day-old mice. Each animal received 0.01 ml intracerebrally, 0.03 ml intraperitoneally, and 0.03 ml intramuscularly of the treated feces. The oral washings were inoculated in the same manner, but the antibiotics were reduced to one-half the quantity. (b) *Virus isolations.* The fecal specimens received were kept frozen in the dry ice refrigerator while all other material was stored in the electric freezer at −10°C. The best results were obtained with oral specimens from patients who had gargled with 40 or 50 ml of tryptose phosphate broth. With specimens obtained early in the illness, the virus was found more often in the oral material than in the feces. The inoculation of 3-to-5-day-old mice with positive human material, produced symptoms of marked ataxia, followed by muscle paralysis progressing to complete immobilization, after which death might occur rapidly or be delayed for several days. Virus was present in the infected mice brains, but a larger amount could be harvested by skinning the animals and removing all skeletal muscles. The muscles were prepared as a 10% suspension in buffered saline contain-

1. Howitt, B. F., Bishop, L. K., and Kissling, R. E., *Am. J. Pub. Health*, 1948, v38, 1263.

2. Howitt, B. F., *J. Immunol.*, 1950, v64, 73.

3. Ginsburg, H. S., and Horsfall, F. L., *J. Exp. Med.*, 1949, v90, 475.

4. Dalldorf, G., and Sickles, G., *Science*, 1948, v108, 61.

5. Gifford, R., and Dalldorf, G., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 589.

6. Howitt, B. F., and Barnett, V. H., *J. Lab. and Clin. Med.*, 1948, v33, 1402.

ing 10% normal rabbit serum and filtered through a Seitz pad. After 2 to 3 passages in mice, muscle filtrates were kept frozen in ampules as the stock virus. (c) *Immune sera.* Immune sera were produced by inoculation of mice, guinea pigs, hamsters, rabbits or monkeys with either the brain or the filtered muscle suspensions. The most effective serum was obtained in about 3 weeks by bi-weekly intravenous injections of rabbits with filtered infected mice muscles. (d) Neutralization tests were performed according to the method in the U. S. Army Laboratory Manual(7) except that the serum-virus mixtures were incubated 20 minutes at 37°C and then inoculated in 0.03 ml amounts intramuscularly into 3-to-5-day-old mice. All sera were inactivated at 56°C for 30 minutes before use. Although the virus strains had LD_{50} titers of between $10^{-6.5}$ and $10^{-8.5}$, many sera gave such high protection that a neutralization index often was not obtained when only 2 or 3 intermediate virus dilutions were used. In tests reported as positive, the serum neutralized at least 1000 LD_{50} doses of virus.

Characteristics of the virus. The strain of Coxsackie virus first isolated came from the feces of a child (B.J.A.) in Shreveport, La., submitted for examination in August, 1948, by Dr. C. H. Webb(8). The patient had a severe frontal headache, fever of short duration, but no other symptoms. The specimen was obtained about 4 days after the onset, and upon inoculation 3 months later, yielded a virus that was fatal for 3-to-7-day-old mice and hamsters but not for 14-day-old mice, guinea pigs, rabbits, chickens, embryonated eggs or monkeys (including one 4-day-old monkey). The virus easily passed through a Seitz filter. It could be transmitted by the intracerebral, intraperitoneal, intramuscular, subcutaneous and intranasal routes of inoculation. It was recovered from the blood stream, the brain, cord, lungs, liver, spleen, kidneys, and intestinal contents of the infected mice,

TABLE I.
Summary of Isolations of Coxsackie Viruses from Human Sources.

State	Oral washings		Feces		Blood		Brain or cord		Spinal fluid	
	No. tested	No. positive	No. tested	No. positive	No. tested	No. positive	No. tested	No. positive	No. tested	No. positive
Ala.*	34	24	16	5	9	8	5	3		
Colo.			2	2			1	0		
Del.	48	7	31	9					20	0
Fla.	3	3	5	5	2	2				
Ga.	10	6	10	5	2	2				
La.	6	2	4	1	1	1	3	2		
Okla.	1	1	4	1	1	1				
S.D.							1	1		
Tenn.	16	5	6	1	10	8	4	2		
Totals	118	48 (40.6%)	78	29 (37.1%)	25	22 (88.0%)	14	8 (57.1%)	20	0

* Virus was recovered from one urine specimen.

7. Simmons, J. S., and Gentzkow, C. J., Lea & Febiger, 1944, Philadelphia.

8. Webb, C. H., Wolfe, S. G., and Howitt, B. F. Presented at the Annual Session of Am. Med. Assoc., Atlantic City, N. J., June 8, 1949.

and was present in high concentration in the skeletal muscles. Although virus may be found in the brain and cord of infected mice, the histopathological lesions were confined to the muscles as observed by Dalldorf *et al.*(9), Melnick *et al.*(10), and also by Dr. R. E. Kissling of this laboratory. This virus was not neutralized by immune sera for the viruses of MV and Lansing poliomyelitis, MM, encephalomyocarditis, F.A. and GD VII (Theiler's mouse strains), Newcastle disease and herpes.

Virus isolations from human material. After the original isolations of the Coxsackie virus from the first few specimens, a systematic study was made of all material remaining from the earlier collections beginning with 1947 through 1949. The Coxsackie virus was isolated from human feces, urine, nasopharyngeal or mouth washings, mouth vesicles, nasal swabs, saliva, sputum, serum, whole blood, cord and brain, but not spinal fluid that had been sent in at various times and from scattered localities in 9 states. All material had been kept frozen at either –10°C or –60°C, and in many instances the virus remained viable after storage for over 2 years at the latter temperature.

Table I gives a summary of 108 isolations of the Coxsackie group of viruses and shows both the geographical location from which the material came and the number of isolations from the different kinds of specimens. While it is believed that this virus is associated with a benign type of infection, it is interesting to note that the virus has been obtained from the nasopharyngeal washings of 1, the serum of 2, and the nervous tissues of 8 fatal cases as shown in Table II. Five of these 10 fatal cases were diagnosed as poliomyelitis. Poliomyelitis and Coxsackie viruses were recovered simultaneously from the spinal cord of one of these patients.

Virus was recovered from either clotted blood or serum of 22 patients. All of the

TABLE II.
Coxsackie Virus Isolations from Fatal Cases.

Case No.	Age, yr	Location	Date of onset	Date of death	Material tested	Diagnosis	Type of virus isolated
1	56	Ala.	1/31/49	2/14/49	Brain	Tuberculosis of lungs—encephalomyelitis	2
2	4	,,	9/ 1/49	9/ 5/49	Spinal cord	Poliomyelitis—Polio virus isolated	4
3	2	,,	9/ 3/49	9/27/49	,, ,,	Autopsy findings—negative	4
4	6	Ga.	5/30/49	6/ 2/49	Serum	(?) Mumps encephalitis	2
5	2	La.		9/ 1/49	,,	Poliomyelitis	2*
6	34	,,	8/ 3/49	8/11/49	Brain	Poliomyelitis or encephalomyelitis	4
7	3	Tenn.	7/15/48	7/25/48	Nasal washing	Encephalomyelopathy	4*
					Brain		4*
					Serum		4
8	34	,,	11/16/49	11/23/49	Brain stem	Poliomyelitis	
9	13	La.	10/ 1/49	10/18/49	Brain	Encephalitis (?)	5
10	20	S.D.	12/10/49	12/17/49	Brain	Poliomyelitis encephalitis	4

* Virus of the same type was isolated a second time from the same material.

9. Dalldorf, G., Sickles, G. M., Plager, H., and Gifford, R., *J. Exp. Med.*, 1949, v89, 567.

10. Melnick, J. L., Shaw, E. W., and Curnen, E. C., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 344.

blood samples had been kept frozen at −10°C and some were held for over a year. While 14 of the specimens were obtained during the first week of illness, one was taken in 9 and another in 13 days after the onset. The date of onset was unknown on 6 of the patients.

As seen in Table III the Coxsackie virus has been recovered from human material obtained in several outbreaks which, although characterized by diverse clinical pictures, were all suspected of having a viral origin. In children, the picture was usually that of a non-paralytic poliomyelitis or a mild 3-to-5-day fever accompanied by headache and occasionally muscle pains; in adults, it was associated often with an influenza-like syndrome. The largest number of isolations came from material of patients ill during August or September. This observation has been reported also by Curnen, Shaw and Melnick(11). Another peak appeared in March in Montgomery when an outbreak in adults was studied at a military base. Here the patients had an influenza-like disease.

Differentiation of the Coxsackie viruses into serological types. Early in these studies it was observed that the antiserum from one strain of virus did not afford protection against two others. When more viruses were isolated, it became apparent that they belonged to several distinct serological types. The presence of a multiplicity of strains for this virus has already been reported by other workers (7,9,12). Through the courtesy of Dr. G. Dalldorf of the New York State Laboratory, antisera were received for viruses which he has designated as Types 1, 2 and 3. By means of both neutralization and complement fixation tests(13), it was found that certain of the strains isolated in this laboratory fell into Types 1 and 2, but none so far into Type 3. In addition, 2 other immunologically distinct viruses have been found that are tentatively called Types 4 and 5 until they can be checked against new strains isolated by other

TABLE III. Outbreaks Associated with Isolations of Coxsackie Virus.

Location	Year	General type of case	Predominant symptoms	Total No. cases tested	Virus isolations from: Feces No. tested	Feces No. positive	Mouth garglings No. tested	Mouth garglings No. positive	Blood No. tested	Blood No. positive
Wilmington, Del.		Poliomyelitis-like	Fever, headache, some muscle weakness	46	31	9	36 12 contact nurses	1 6		
Enterprise, Ala.	48	,, ,,	3-5-day fever, headache, nausea, vomiting	18	6	2	5	2	7	6
Shreveport, La.	48	Mild fever	3-day fever, headache, no neurological symptoms	5	4	1	6	2		
Nashville, Tenn.	48	Encephalitis-like or atypical syndromes	Varied symptoms, headache, fever	17	3	0	13	3	10	8
Montgomery, Ala.	49	Influenza-like	Fever, aches, some mouth vesicles	21	—	—	21	12		
Swainsboro, Ga.	49	Generalized febrile disease	Fever, headache, vomiting, malaise	14	9	4	9	5	1	1

11. Curnen, E. C., Shaw, E. W., and Melnick, J. L., *J.A.M.A.*, 1949, v141, 894.

12. Sickles, G. M., and Dalldorf, G., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 30.

13. Howitt, B. F., and Benefield, U. R., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 90.

TABLE IV.
Distribution of Virus Types.

State	Type 1*	Type 2*	Type 4‡	Type 5‡
Ala.	1	2	14	8
Colo.			2	
Del.	1	6	3	
Fla.				7
Ga.		1	6	1
La.		3	1	1
Okla.		1	2	
S.D.			1	
Tenn.	1	4	6	1
Total No. typed†	3	17	35	18

* Types 1 and 2: Sickles and Dalldorf(10).
† Each number represents an individual case.
‡ These type numbers are given tentatively pending a cross typing with strains isolated by other workers.

workers. Table IV shows the geographical distribution of the types according to states.

Not all of the viruses isolated have been typed as yet; but thus far, Type 4 has been isolated most frequently, mainly in oral secretions, while Types 5 and 2 are next in prevalence. Type 5 has been recovered most often from Florida and Alabama, while Type 4 predominated in one outbreak in Georgia. So far only Types 2 and 4 have been found in material from fatal cases. Second isolations of virus have been made from 5 of the human tissues, and repeated recoveries have been obtained from the same feces of one patient and the mouth washings of another. Type 4 virus has been found in the brain, oral washings and serum of one person and recovered twice from each of these same specimens, while paired materials (feces and mouth garglings or blood and nasal washings) from 3 individuals have yielded the same type of virus from each pair. The virus could be obtained not only from different tissues or excretions of the same person but also from material taken at different time intervals. Mouth garglings from 4 individuals were positive when obtained 2 weeks after the first isolations. Type 4 virus was isolated 3 times at weekly intervals from one of the patients.

However, there is a doubt as to the exact origin of some of these isolations, because it was found that Type 4 virus could be obtained on 3 occasions from muscle filtrates of suckling mice that had been inoculated with filtered muscles of normal baby mice. These animals had been left for 4 days in the laboratory before they were killed and their muscles passed to other normal mice. Because antibodies for this strain have been found in human sera and because it was first isolated from a human source, it seems more probable that this virus may be disseminated easily in the laboratory rather than that it is normally latent in the mice tissues.

Neutralization tests against the Coxsackie types of virus. The different strains of Coxsackie virus were easily distinguished by means of the serum neutralization test because the reactions were clear-cut when appropriate dilutions of virus were used. Most of the strains had an LD_{50} titer between $10^{-6.5}$ and $10^{-8.0}$, so that when the tests were prepared with undiluted standard sera against dilutions of 10^{-4} and 10^{-5} of the unknown virus, complete protection obtained with only one serum was convincing evidence for the type differentiation. Antibodies against virus Types 1 and 2 were found in human paired sera from one outbreak where both these types of Coxsackie virus had been isolated. Of 13 paired sera tested, 1 was positive for Type 1, 4 for Types 1 and 2, and 6 for Type 2 alone. On 3 occasions only was there a rise in titer between the acute and convalescent sera. Twelve sera of another group showed positive antibodies against 1 or more of the strains, including Types 4 and 5. On 7 occasions antibodies were found in the patient's serum against the same strain of virus isolated from that individual. Positive neutralization tests also were obtained against Type 2 virus in 4 sera from normal individuals. The complement fixation test, using infected muscle as antigen, can also be used effectively for strain differentiation and antibody determination (13). Further serological studies are in progress.

Discussion. The Coxsackie virus has been isolated from sporadic cases and from groups of patients involved in diseases with variable and ill-defined clinical pictures such as mild fevers, poliomyelitis-like or influenza-like syndromes, or occasionally encephalitis. The same virus type predominated in 2 localities, while in others the types were mixed. While

other workers(8,9,10,11,14) have recovered the Coxsackie viruses from cases without sequelae, it is significant that in this study they were found also in the tissues and secretions of 10 fatal cases, 5 of which were diagnosed as poliomyelitis. Poliomyelitis virus was recovered also from the spinal cord of one of these patients and from the feces of 2 recovered cases. Whether or not the Coxsackie viruses were responsible for death is as yet a matter for conjecture. The frequency of isolation of the Coxsackie viruses from the blood stream is significant not only because of the ease with which the material may be inoculated into suckling mice with little preliminary preparation, but also because the blood is thus a potential source of virus for blood-sucking vectors. Melnick *et al.*(10) have reported on the recovery of the Coxsackie virus from flies taken in nature. However, the evidence at present points to direct person to person transmission because of the ease with which the virus may be demonstrated in the oro-nasopharyngeal passages. The isolation of the virus from the mouth washings of a group of 6 out of 12 nurses (Table III) who were asymptomatic but in contact with patients having illnesses probably due to this virus favors the respiratory route of spread. The discovery of this new group of viruses with its widespread prevalence may require a revision of our epidemiological thought, especially in regard to nonparalytic poliomyelitis and other minor illnesses. The correct interpretation of the association of these viruses with such clinical syndromes has yet to be made, but further studies will no doubt elucidate its true significance.

Summary. One hundred eight isolations of the Coxsackie group of viruses have been made from the secretions or tissues of 97 patients from 9 different states with multiple isolations from different kinds of specimens from 6 separate individuals. Virus was recovered a second time from 9 different specimens. The strains obtained may be divided into 4 distinct serological types. Two of them correspond to Types 1 and 2 of Dalldorf; the other 2, tentatively called Types 4 and 5, are antigenically different. The viruses have been recovered from human blood, serum, brain, cord, mouth or nasopharyngeal washings, saliva, sputum, nasal swabs, mouth vesicles, urine and feces with a predominance of Types 4, 5 and 2, respectively. The highest percentage of isolations was from the blood, 22 out of 25 (88.0%) specimens being positive. The virus was found also in material from 10 fatal cases and could be recovered repeatedly from 5 of the same specimens. It was easily isolated from mouth washings during the first week of illness, and on 3 occasions was obtained again from the same patients during the third week after the onset. The largest number of isolations was from material obtained during August and September, with another large group collected in March. Neutralizing antibodies for Types 1 and 2 were found not only in the sera of patients but in those of a few normal individuals as well. The sera of 7 out of 8 individuals showed neutralizing antibodies against their homologous viruses. Type 4 virus has been recovered 3 times from muscle filtrates of normal baby mice and there is evidence that the Coxsackie viruses may be disseminated in the laboratory, thus accounting for some of the isolations.

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14. Jaworski, A. J., and West, E. J., *J.A.M.A.*, 1949, v141, 902.

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