the radioactivity recovered in the 24-hour period following infusion of the 4 subjects. The amount of VA was not determined for each individual.

After acidification and extraction with ethylacetate of the radioactive material eluted with Carrier VA using Procedure II, it was found that the major portion of this radioactivity co-chromatographed with Carrier VA in the 3 different solvent systems used (Fig. 1). Solvent Systems A and B are extremely useful in separating free phenolic acids such as MOMA, DOMA, and VA since additional hydroxy groups, whether aromatic or aliphatic, markedly slow down the rate of movement(7). The chromatographic peak from System C (Fig. 1), containing Carrier VA, as indicated by optical density, and radioactivity, was collected, adjusted to pH 6.1, placed on a Dowex column and subjected to gradient elution (400 ml total volume; 100 ml per reservoir). The presence of VA-C^{14} is indicated by the elution pattern of the radioactivity and Carrier VA from this column (Figure 2).

Discussion. The ultimate formation of a small amount of VA from noradrenaline involves oxidative degradation of the side chain; therefore, the most likely immediate precursor is MOMA. The recent findings(8) that the perfusion of rat liver with MOMA led to formation of VA supports this concept; however, Pellerin and D'Iorio(7) found that incubation of DOMA with rat liver or kidney slices led to formation of both MOMA and VA; thus, it is possible that DOMA may undergo degradation to 3,4-dihydroxybenzoic acid which could then be methylated to yield VA. Two other compounds formed from noradrenaline(9), 3-methoxy-4-hydroxy-mandelaldehyde, and 3-methoxy-4-hydroxyphenylethylglycol, could also be degraded to VA. The contribution of the various possible pathways leading to VA requires further study.

Summary. VA has been identified as a urinary catabolite of noradrenaline in man. In the 24-hour period following infusion of dl-noradrenaline-2-C^{14} into 4 subjects, the urinary VA accounted for 2.6% of the recovered C^{14}.

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The Price Virus. An Unclassified Enterovirus Isolated from Patients with Central Nervous System Disease.* (27647)

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Over the past 5 years viral isolation attempts on stool specimens from patients with symptoms of CNS disease have led to the recovery of 6 immunologically related strains of virus which appear to be distinct from any of the currently recognized enterovirus types. This report describes certain properties of these viruses and presents evidence confirming their human origin.

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Materials and methods. Viral isolation attempts. All of the isolates were recovered in

monkey kidney cell cultures prepared by a slight modification of the method of Bodian (1); cultures were maintained on Eagle's medium without serum. The preparation of stool suspensions and inoculation of cell cultures have been described(2).

Preparation of HeLa cell cultures. Tube and bottle cultures of the "D" line of HeLa cells were initiated and maintained as described elsewhere(2). For preparation of complement-fixing antigens, bottle cultures of HeLa cells were maintained on a medium consisting of 2.5% inactivated monkey serum, 47.5% mixture 199 and 50% lactalbumin hydrolysate-yeast extract medium(2).

Preparation of immune sera. Immune sera to the 3 poliovirus types, Coxsackie group B types 1-6 and group A type 9, the 28 ECHO virus types, 4 ECHO candidate strains and 2 of the viral isolates under study (Price and Watts) were prepared in monkeys(3). Immune sera to group A Coxsackie virus types 1-24 were produced in hamsters(4), and immune sera to 4 of the strains under study were prepared in hamsters by inoculating the animals intraperitoneally with 2 ml of a mixture of equal parts of infected monkey kidney tissue culture fluid and adjuvant.† The animals received 3 inoculations at weekly intervals and were bled 7-14 days after the last immunizing injection.

Procedure for identification of polioviruses, Coxsackie viruses and ECHO viruses. The viruses recovered in monkey kidney cell cultures were tested against immune sera to the 3 poliovirus and the 6 group B Coxsackie virus types by a colorimetric neutralization technic employing the FL line of human amnion cells(5). Isolates not identifiable in this system were tested in monkey kidney cell cultures against immune sera to the currently recognized ECHO viruses and Coxsackie type A9 virus. If viral strains were pathogenic for suckling mice they were tested in this system against immune sera to Coxsackie group A viruses.

To detect possible mixtures of viruses, isolates which were not neutralized by immune sera to any of the known enteroviruses were plaque-purified 2 times in monkey kidney cells and then retested against enterovirus immune sera.

Viral strains were carried through 2 plaque purifications before they were employed for preparation of immune sera.

Neutralization tests. The procedures for neutralization tests in monolayer cultures of monkey kidney and HeLa cells have been described in detail(2,3,6). HeLa cell-adapted virus was used for colorimetric neutralization tests conducted in the same manner as tests for certain ECHO viruses(7).

Complement fixation (CF) tests. The standardization of complement-fixing antigens (8) and performance of CF tests(9) have been described.

Hemagglutination (HA) tests. Viral strains were examined for possible hemagglutinating activity by the method of Goldfield *et al.*(10).

Results. Clinical remarks. Table I presents data on the isolation of the 6 viral strains from patients with CNS disease, and data on a sibling with no viral isolation, but whose serum specimens showed a 4-fold rise in neutralizing antibody to the Van Gorkam virus strain. Each of the patients experienced an illness which could be clinically characterized as a mild to moderately severe aseptic meningitis syndrome. However, in 2 instances (a 2-year-old boy (case No. 5) and his 8-year-old sister (case No. 7)), the presence of muscle spasm and apparent weakness during the acute phase of illness led to an initial diagnosis of paralytic poliomyelitis; complete recovery ensued and no residual weakness was demonstrable 5 months later.

Fever and headache were common to all of the cases and vomiting occurred in 4. Nuchal rigidity was definitely demonstrable in only 3 instances, being questionable or absent in the remainder. Spinal fluid examination showed elevated leucocyte counts ranging from 13 to 460 cells, varying widely in the proportion of lymphocytes (from 20 to 80%).

Cytopathic effect of viral strains in tissue cultures. As indicated above, isolation of the 6 viral strains was accomplished in monkey kidney cell cultures. However, the strains

† Adjuvant consisted of one part of Arlacel A and 9 parts of Standard mineral oil, C.T. 70.

TABLE I. Recovery of Price Virus and Immunologically Related Strains from Patients with CNS Disease.

Patient	Age, yr	Sex	Date of onset	Clinical diagnosis	Virus isolation from stools: Days after onset	Virus isolation from stools: Results
1. Nunez	8	♀	3/25/57	Aseptic meningitis	8	+
					12	0
2. Sievers	12	♂	8/19/59	" "	6	+
3. Price	8	♂	9/24/59	" "	4?	+
4. Watts	17	♀	11/29/59	" "	3–9?	+
5. Van Gorkam, J.	2	♂	8/ 4/60	Paralytic poliomyelitis	5	0
					6	+
6. Torwick	7	♀	12/ 3/61	Aseptic meningitis	2	+
					4	0
					5	+
7. Van Gorkam, P. (sibling of Van Gorkam, J. 4× rise neut. antibody to his viral strain)	8	♀	8/ 2/60	Paralytic poliomyelitis	7	0
					8	0

were slow to establish themselves in this cell type; on first passage all 6 showed only a questionable or minimal cytopathic effect (CPE) after 7 days' incubation, and on second passage only a minimal CPE was seen. The strains required 3-5 passages in monkey kidney cell cultures before they could be considered well established in the tissue culture system. They all produce a "characteristic enterovirus CPE" in monkey kidney cells (Fig. 1).

Even after multiple passages in monkey kidney cell cultures there was considerable variation in the infectivity titers of the different strains in this cell type. The Watts strain had the highest titers ($10^{-5.5}$-$10^{-6.5}$), the Price and Nunez strains intermediate titers ($10^{-4.0}$-$10^{-4.5}$) and the Torwick, Van Gorkam and Sievers strains had low titers ranging from $10^{-2.5}$ to $10^{-3.5}$. Efforts to improve titers by further passages in monkey kidney cells were not successful.

All 5 strains on which plaque purification was done produced distinct plaques 2-5 mm

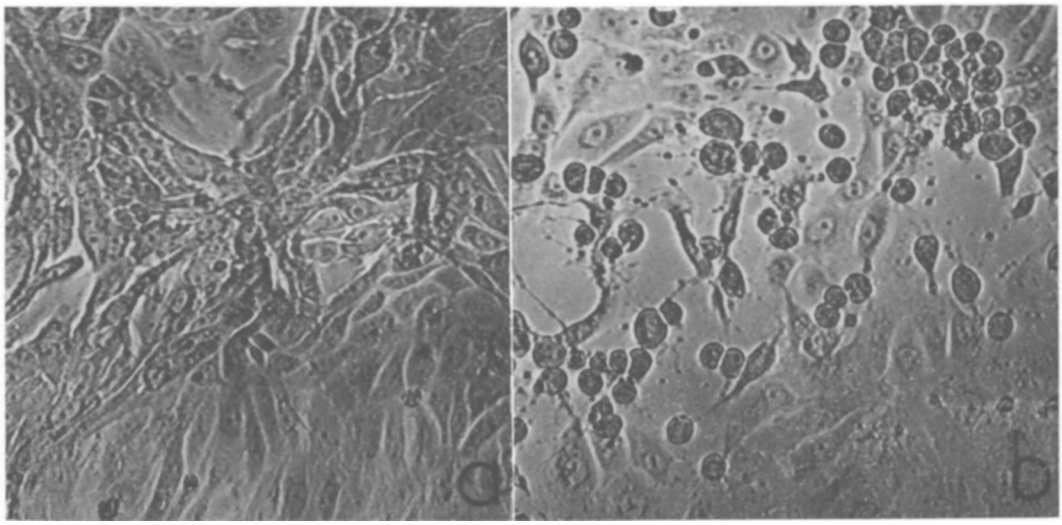

FIG. 1. Cytopathic effect of Price virus in monkey kidney cell cultures. *a*. Uninfected monkey kidney cell culture. *b*. Monkey kidney cell culture 5 days after inoculation with 100 TCD_{50} of Price virus.

in diameter in monkey kidney monolayers after 4-5 days' incubation.

When low monkey kidney passage material of the several strains was inoculated into FL amnion cells CPE was not observed, and attempts to reisolate some of the strains in HeLa cells were not successful. However, after the strains were established and plaque-purified in monkey kidney cells they were easily adapted to growth in HeLa cells of the "D" line. On first passage of undiluted, infected monkey kidney tissue culture fluid into HeLa cells, the CPE was not distinct, but on second passage a clear-cut cytopathic effect was seen with all strains. After a few passages most of the strains showed higher infectivity titers ($10^{-5.0}$-$10^{-6.0}$) in HeLa cells than in monkey kidney cell cultures.

Non-pathogenicity of the viral strains for suckling mice. The stool suspensions from which the Nunez and Sievers strains were recovered were inoculated into suckling mice less than one day of age(6) with negative results. Infected monkey kidney and HeLa cell fluids representing the 6 strains also failed to produce overt illness in suckling mice.

Attempts to detect occurrence of hemagglutinins. The procedure for hemagglutination (HA) tests with the ECHO viruses(10) was used to assay monkey kidney passage material of all 6 viral strains for possible HA activity, but hemagglutination of human group O, chicken or guinea pig erythrocytes was not observed at 4°C, room temperature, nor 37°C. In view of the recent demonstration that hemagglutinins of Coe virus (an enterovirus (11)) may be "unmasked" by fluorocarbon (12), fluids from monkey kidney cell cultures infected with the 6 viral strains were treated with this reagent; no hemagglutinin could be detected in the treated fluids.

Attempts to identify the viruses with currently recognized enteroviruses. Table II summarizes the results of neutralization tests conducted to determine whether the 6 viral strains represented any of the currently recognized enterovirus immunotypes. HeLa cell-adapted viruses were employed for colorimetric tests (in HeLa "D" cells) against immune sera to the 3 poliovirus and 6 group B Coxsackie virus types. None of the sera neutralized any of the strains, but all were neutralized, in the same run, by monkey immune serum to the Price virus strain.

The virus strains were not neutralized by monkey immune sera to any of the 28 ECHO virus types or 4 ECHO candidate virus strains (Caldwell, JV-6, Taylor, and Wilt).‡ Although no evidence was obtained that the viruses were pathogenic for the suckling mouse,

TABLE II. Neutralization Tests Conducted in Identification of Price Virus and Related Strains.

Virus strains tested	Type of neut. test	Immune sera employed	Results
Price, Watts, Van Gorkam, Torwick, Sievers, Nunez	Colorimetric* in HeLa cells	Poliovirus, types 1-3	Neg.
Idem	*Idem*	Cox. group B, types 1-6	"
"	Tube neut., MK† monolayers	Cox. B6	"
"	*Idem*	Cox. group A, types 1-24	"
"	"	ECHO virus, types 1-28	"
"	"	ECHO virus candidates strains: Caldwell, JV-6, Wilt, Taylor	"
"	"	Price	Pos.
"	Tube neut.,* HeLa cell monolayers	"	"
"	Colorimetric* in HeLa cells	"	"

* HeLa cell-adapted strains of virus employed.

† MK = monkey kidney cells.

‡ The JV-6 and Taylor viruses have since been shown to be related to JV-10 virus, the prototype of enterovirus 59; the Wilt virus has been found to be related to ECHO 20 virus.

they were tested against hamster immune sera to the 24 currently recognized immunotypes of Coxsackie A virus to rule out the possibility that they might be strains of Coxsackie A virus, but lacking in mouse pathogenicity (11). The results of these tests were also negative.

Thus, the 6 viral strains appeared immunologically distinct from any of the currently classified enteroviruses.

Immunologic relationship of the 6 strains to each other. Because the Watts strain showed the highest titers in monkey kidney cell cultures it was initially chosen for preparation of immune sera to be used for possible identification of other isolates. When the 6 strains were tested against immune serum to the Watts virus, neutralizing activity against all of the strains was noted, but the neutralizing capacity was quite low with certain of the strains. The possibility of immunologic variation between these 6 strains was further investigated, and it would appear that they are all immunologically related, but not strictly identical.

Table III presents results of cross neutralization tests with hamster immune sera to 4 of the viral strains. The Watts virus would appear to possess a narrow antigenic spectrum in that it was easily neutralized by immune sera to other strains, but Watts immune serum had a low neutralizing titer for certain strains. The Price virus, on the other hand, appears to have a broad antigenic spectrum, as immune serum to this strain displayed good neutralization titers for all 6 viral strains. Immune sera to the Van Gorkam and Torwick strains had essentially similar titers for all of the strains except the Nunez strain, which would appear to be more closely related to the Watts strain than to the others (see below).

TABLE IV. Neutralization Tests with Price Virus and Related Strains against Monkey Immune Sera to Price and Watts Virus.

Test virus, strain (100 TCD_{50})	Neutralization titers of monkey immune sera to	
	Price virus	Watts virus
Price	1:256	1:32
Watts	1:256	1:1024
Van Gorkam	1:256	1:128
Torwick	1:64	1:32
Sievers	1:256	1:32
Nunez	1:64	1:1024

Table IV gives results of cross neutralization tests with monkey immune sera to the Price and Watts viruses, and again it is seen that the Price virus immune serum had neutralizing titers differing by no more than 4-fold against all 6 strains, while the Watts immune serum showed low neutralizing titers for the Price, Torwick and Sievers strains, an intermediate titer for the Van Gorkam virus and a high titer for the Nunez and Watts strains.

Because of its broader antigenic spectrum the Price strain was chosen as the representative strain for this group of 6 isolates.

Neutralization tests with patients' sera and their infecting viral strains. To establish the human origin of the viral strains, the sera of patients from whom they were isolated were examined in neutralization tests against their infecting viral strains and the Watts strain. Results are presented in Table V. Five of the 6 patients showed diagnostically significant rises in neutralizing antibody titer against both their infecting strain and the Watts strain. (Although patient No. 2 failed to show neutralizing antibody for either strain, the virus was successfully reisolated from his stool specimen.) Of note is the fact that a

TABLE III. Cross Neutralization Tests with Price Virus and Related Strains.

Test virus, strain	Test dose of virus	Neutralization titers of hamster immune sera to			
		Price virus	Watts virus	Van Gorkam virus	Torwick virus
Price	100 TCD_{50}	*1:256*	1:32	1:128	1:64
Watts	100 "	1:256	*1:256*	1:256	1:128
Van Gorkam	100 "	1:256	1:32	*1:256*	1:128
Torwick	100 "	1:128	1:32	1:128	*1:128*
Sievers	30 "	1:256	1:16	1:256	1:128
Nunez	100 "	1:128	1:256	1:32	1:16

TABLE V. Evidence for Human Origin of Price Virus and Related Strains. Neutralization tests on sera from patients infected with the viral strains.

Patient	Days after onset	Neut. antibody titer against 100 TCD_{50} of Infecting virus strain	Watts strain
1. Nunez	8	<1:8	<1:8
	32	1:32	1:8
	58	1:32	1:16
2. Sievers	3	<1:8	<1:8
	23	<1:8*	<1:8
3. Price	4	<1:8	<1:8
	18	1:16	1:16
4. Watts	3		<1:8
	20		1:32
5. Van Gorkam, J.	1	<1:8	<1:8
	30	1:64	1:64
6. Torwick	2	<1:8	<1:8
	16	1:16	1:16
7. Van Gorkam, P.†	6	<1:8‡	
	20	1:16	
	34	1:16	

* Although no antibody was demonstrable, reisolation of the virus from original stool specimen was accomplished.

† Sibling of Van Gorkam, J., ill at same time with clinical symptoms of paralytic poliomyelitis, no virus isolation and negative poliovirus serology.

‡ Neut. tests conducted with J. Van Gorkam strain.

sibling (patient No. 7) of one of the patients with a viral isolation (patient No. 5) showed a significant antibody rise to the virus. These children were ill at approximately the same time with clinical symptoms of paralytic poliomyelitis (Table I), but poliovirus was not recovered from their stools, and there was no serological evidence of a current poliovirus infection.

Complement fixation reactions with the 6 strains. Further evidence of the immunologic relationship of the 6 strains was obtained by complement fixation tests. Antigens were prepared for each strain in HeLa cell cultures (8) and tested in block titrations against monkey immune sera to the Price and Watts strains. Table VI shows that the immune sera had similar CF titers against 2 units of each strain antigen. The antigenic differences observed between strains in neutralization tests were not so apparent in CF tests, as all the strains fixed complement to a high titer with monkey immune sera to both the Price and Watts viruses. The Price antigen did not fix complement with monkey immune sera to ECHO virus types 1-28; Coxsackie B virus, types 1-6; or Coxsackie A virus, type 9.

Ether resistance of Price virus. Infected monkey kidney and HeLa cell culture fluids were mixed with ethyl ether at a concentration of 20% and held at 4°C for 18 hours. Parallel titrations showed no difference in the titers of ether-treated and untreated fluids, indicating that the Price virus possesses the enterovirus characteristic of ether resistance.

Size of the Price virus. Filtration through Gradocol membranes was done to determine the approximate size of the Price virus, and whether the size falls within the enterovirus range. In 3 different experiments it was found that essentially all of the infectious virus was retained by filters with an average pore diameter (APD) of 38 mμ; applying Elford's(13) conversion factor of 0.5 for small viruses, this would indicate an average size of approximately 19 mμ.

Electron micrograph size determinations of the Price virus were recently performed in this laboratory by Dr. L. H. Frommhagen. Virus propagated in HeLa cells was purified in a cesium chloride gradient of density 1.34 (14). Electron micrographs of spray droplets of the purified preparation showed a particle diameter of 29 $\pm$ 4 mμ.

Discussion. The 6 viral strains described herein possess characteristics which would classify them as human enteroviruses, *i.e.*, their recovery from human stool specimens (and rises in titer of specific neutralizing antibody in the patients from whom the recoveries were made), ether resistance, particle size, and a typical "enterovirus cytopathic effect" in monkey kidney cell cultures. The

TABLE VI. Complement Fixation Tests with Price Virus and Related Strains.

Viral strain employed for preparation of CF antigen	Titer of Price monkey immune serum *vs.* 2 units of CF antigen	Titer of Watts monkey immune serum *vs.* 2 units of CF antigen
Price	1:512	1:512
Watts	1:512	1:512
Nunez	1:256	1:256
Torwick	1:512	1:512>
Van Gorkam	1:256	1:512
Sievers	1:512	1:512>

representative Price strain has been submitted to the Enterovirus Committee as a candidate for classification as an ECHO virus immunotype.

The several viral strains apparently represent an immunotype containing "prime" strains. The low neutralizing capacity of certain immune sera for heterologous strains does not appear to be similar to the "broad and specific" phenomenon observed with ECHO 6 viruses(15) in which newly isolated strains are poorly neutralized, but with continued tissue culture passage become easily neutralizable. The Price virus and related strains were studied at relatively high passage levels, and still displayed consistent variations with respect to neutralization. Results of complement fixation tests with immune monkey sera confirm the antigenic relationship of the 6 virus strains to each other.

Although these limited observations do not prove an etiologic relationship of the Price virus to central nervous system disease, the recovery of virus during the first few days of illness and the demonstration in the patients of significant rises in neutralizing antibody level indicates that infections with the Price virus were temporally associated with the illnesses in question. The repeated observation of this association in sporadic cases of aseptic meningitis over a period of 5 years is strongly suggestive of a causal relationship.

Further studies on the incidence of antibody rises to Price virus in the sera of CNS disease patients for whom it has been impossible to obtain laboratory evidence of other viral infections would be of interest. The fact that the agent is slow to establish itself in monkey kidney cell cultures may account for the low number of isolates recovered from testing of hundreds of stool specimens.

Summary. Six immunologically related viral strains have been recovered from the stools of patients with CNS disease. Neutralization tests indicate that they are not polioviruses, group A or group B Coxsackie viruses, ECHO virus types 1-28 or one of 4 ECHO candidate strains. The human origin of these agents is evidenced by the fact that 5 of the 6 patients with isolations showed 4-fold or greater rises in neutralizing antibody to these viruses. The several strains possess the characteristics of human enteroviruses, *i.e.*, ether resistance, particle size, and production of a characteristic "enterovirus cytopathic effect" in monkey kidney cell cultures. All of the strains could be adapted to growth in HeLa cells. They are not pathogenic for suckling mice, and do not agglutinate human group O, chicken or guinea pig erythrocytes.

ADDENDUM. Since this paper was submitted for publication, information from Dr. H. A. Wenner, as well as the results of tests performed in this laboratory, indicates that the Bastianni, PR-17 and Frater viruses (enterovirus candidates) are related to the Price virus.

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