the population. Certainly, this explanation is substantiated by the difference noted in the response of the L-97 pool and plaque 29 pool with regard to their growth potential at 40°C following cold passage. The difference was probably related to the fact that the entire L-97 pool contains particles that have good growth potential at 23°C and little or none at 40°C. In contrast, there were no particles of this type in the unpassed material from genetically homogeneous plaque 29.

The importance of passage in the cold is emphasized by the development of strains with more stable genetic characteristics and with less neuropathogenicity following passage at 23°C(6,7). The relationship between cold passage and stability is not clearly understood. However, reduction in the number of particles with growth potential at 40°C is probably an important factor.

Summary. The reproductive capacity of several type 3 poliovirus strains was studied at 23°C, 37°C, and 40°C. There was marked variation in the capacity of the strains to replicate at different temperatures. Two wild strains, L-97 and L-38, were serially passed at 23°C. After 14-16 passages at 23°C, each strain lost its growth potential at 40°C while its growth potential at 23°C increased. A plaque pool obtained from the L-97 strain had the interesting ability to replicate well at all 3 temperatures. This plaque pool did not entirely lose its growth potential at 40°C even after 16 passages at 23°C. A plaque obtained from L-38 was passed at 23°C. There was a marked alteration in reproductive capacity at various temperatures of this particular plaque pool during a single passage, the third, at 23°C.

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Received October 22, 1962. P.S.E.B.M., 1963, v112.

Modification of an Attenuated Type 2 Polio Vaccine Following Passage at 23°C.* (28008)

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The passage of poliovirus strains in tissue culture maintained at low temperatures of incubation has been shown to have a marked effect upon the genetic characteristics of a virus population(1,2). The establishment of a genetically stable type 3 poliovirus strain (WM-III) following serial passages of an unstable strain at low temperatures has focused attention upon the possibility of applying the cold-passage technic to other strains that appeared to have potential as live attenuated poliovirus vaccines. Experiments in this area have value in the possible development of new strains or modification of existing strains. In addition, the theoretical aspects of the study of cold-passage would be aided by establishing the frequency of occurrence of both the stabilizing and the attenuating effects previously observed.

* This work was supported (in part) by a USPHS research grant from Nat. Inst. of Allergy & Infect. Dis.

TABLE I. Monkey Neurovirulence of the W-II Strain and of Several W-II Progenitor Strains.

Virus strain	Passages in tissue culture	Dose/monkey in $\log_{10}$ TCD_{50}	Paralyzed	With lesions
TN	0	3.5*	0/4	1/4
TN	4 at 37° & 1 PP†	4.5–4.7	3/11‡	8/11‡
W-II		5.9	0/10	0/10

* Dose was determined by titration in mice.
† PP = Serial single plaque passages on monkey kidney tissue culture monolayers. Three plaques were picked and tested (see Fig. 1).
‡ Cumulative results obtained from 3 separate plaque pools.

For the passage series described here a type 2 strain, TN, was chosen. The TN strain was developed by passage of material obtained from an asymptomatic case of poliomyelitis in cotton rats and mice(3,4). This strain was the first of the experimental oral polio vaccines and was used extensively in human feeding experiments between 1950 and 1953(3,4,5,6). The virus had a high rate of infectiousness for man and produced excellent antibody response (85% of those fed had a marked increase in antibody titer against type 2 viruses). Subjects fed the TN strain have maintained high antibody levels against type 2 viruses for more than 10 years after administration of the vaccine(7). The pool used in the above vaccine program was prepared from mouse brain material. To obtain larger quantities of vaccine material and to plaque purify the strain, attempts were made during the above-mentioned period to adapt the TN strain to monkey kidney tissue culture (MKTC).

Initial passage in the tissue culture system did not yield any cytopathic effects. By the third passage, however, cytopathic changes, limited in extent but typical of poliovirus infection, were seen. The virus material obtained after the fourth passage in MKTC at 37°C (by which time the strain had completely adapted to growth on MK) was tested for its pathogenicity in monkeys by intracerebral inoculation. The tissue culture adapted strain of TN produced more extensive neuropathology than the original mouse adapted strain (Table I) and, therefore, was unsuitable as a vaccine. Also, certain studies indicated that the mouse adapted TN strain, similar to the progenitor of the WM-III strain, was unstable during human passage(6). Because of the excellent vaccine potential (high infectiousness and long-lasting immune response) exhibited by the mouse adapted TN strain, an attempt was made to reduce the pathogenicity for monkeys that had been obtained following passages in MKTC and to improve the strain's stability. For this purpose, cold-passage in tissue culture systems was employed.

Materials and methods. Tissue culture systems. Monolayer cultures of primary trypsinized monkey kidney cells (obtained from Microbiological Associates, Bethesda, Md.) were prepared by adding 10^6 cells suspended in 5 ml of Earle's salt solution containing the mixture of amino acids and vitamins recommended by Eagle(8) (Eagle's in Earle's medium) and 10% calf serum to 60 mm Petri dishes. The Petri dishes were incubated at 37°C in an atmosphere of 4% CO_2 in air. Continuous monolayers were formed after 6-8 days of incubation.

Monkey kidney tube cultures were prepared by seeding each tube with 3×10^5 cells suspended in 0.5 ml Hanks' basic salt solution (BSS) containing 0.5% lactalbumin hydrolysate and 2.0% calf serum in Hanks' BSS. After 4 days incubation the medium was changed to Earle's BSS containing 0.5% lactalbumin hydrolysate and 2.0% calf serum. Before inoculation of the tubes with virus, this medium was replaced with 0.9 ml of Eagle's medium in Earle's salts for maintenance. Streptomycin (100 μg/ml) and penicillin (100 units/ml) were included in all media used for cell growth or maintenance in these experiments.

Tube cultures were used for virus passage and for titration of virus preparations at different temperatures of incubation. For passage in tube cultures, 3 tissue culture tubes were inoculated with 0.1 ml of undiluted supernatant fluid containing virus from the previous passage. After incubation for 1 hour at room temperature, the fluid containing virus was removed, the monolayer cultures washed once with Hanks' BSS and fresh maintenance medium (Eagle's in

Earle's) added. The tubes were incubated at the specified temperature until at least 50% cellular degeneration was noted. The contents of the tubes were frozen and thawed 3 times and pooled; this material was then used as seed virus for the next tissue culture passage. Titrations in tube cultures were done by adding 0.1 ml of each of the desired dilutions to 3 tubes. Dilutions were made in Hanks' BSS. Virus titers were determined by the 50% end-point method(9).

Monolayer cultures in Petri dishes were used for plaque isolations. Virus was allowed to adsorb for one hour. The monolayers were then washed once with Hanks' BSS and overlaid with 5 ml of an agar overlay medium that has been described elsewhere(10). On the second day after inoculation, 2 ml of a second agar overlay containing neutral red at a concentration of 1:13,000 were added. Plaques were observed from the 3rd to the 7th day of incubation. Plaques were picked at random, dispensed into 1 ml of Eagle's in Earle's medium, and stored at −20°C.

One of the vaccine pools used in this study was prepared from monolayer cultures of a human fetal diploid cell strain, WI-26, in continuous culture(11). The WI-26 cell strain was grown in 1, 2 and 5 liter bottles in Eagle's in Earle's medium supplemented with 10% calf serum. Vaccine pools were produced in WI-26 cells by technics previously outlined(12).

Temperature control. Incubators (Assmundson Aktiebolag-Assab Bacteriological Cabinet T-200), equipped with fans to insure a uniform temperature throughout, were kept at 23, 37, and 40°C for incubation of plate cultures. Thermometers were used to check the temperature which varied by ± 0.1°C. Culture tubes were kept in water baths equipped with a heating and agitating apparatus that maintained the temperature to within ± 0.02°C of the desired value (Mergotherm, H. Struers Chemiske Laboratories).

Monkey virulence tests. Cynomologous monkeys weighing 2.5 to 4 kg were inoculated with 0.5 ml of virus suspension into each thalamic area by means of a No. 24 (1¼″) needle. The animals were then observed daily for evidence of paresis or paralysis and after 18-21 days were sacrificed. Histological examination of the formalin-preserved brain and spinal cord tissue began with sectioning of the thalamus to demonstrate the needle tract. If the tract was found, blocks were removed from the midbrain, medulla, cervical enlargement, and lumbar enlargement, and embedded in celloidin. After being sectioned and stained with thionine, every 10th section was examined microscopically for the presence of inflammatory lesions and neuronal destruction or other histological evidence of poliomyelitis infection.

Experimental. History of passages. Fig. 1 summarizes the schedule of tissue culture and plaque passages leading to development of the W-II strain. The TN strain that was the progenitor of the passage series had been developed by 29 serial passages in mice. This strain was tested for neurovirulence in monkeys by intracerebral inoculation (Table I). None of the 4 monkeys injected showed paralysis and only 1 of the 4 had histologic changes. In contrast, 3 plaque purified clones obtained from a 4th MKTC passage pool yielded a significant percentage of paralysis and extensive histopathology. Because of this increased pathogenicity and in view of the results obtained with the cold passage series that had led to the WM-III strain, it was decided that adaptation of the TN strain to the MKTC system should be accompanied by passage at low incubation temperatures. This led to the series of cold-passages summarized in Fig. 1. The product of this passage series, the W-II strain, was tested for neurovirulence in monkeys (Table 1). None of the 10 monkeys inoculated showed any signs of paralysis. Also, examination of over 150 sections of central nervous system from each monkey failed to reveal histologic changes in any of the monkeys.

Reproductive capacity temperature tests of W-II strain, several W-II progenitor strains, and MEF-1 strain. Virus pools obtained from several passage levels of the TN strain were tested for their reproductive capacity temperature/40 (rct/40) character by dual

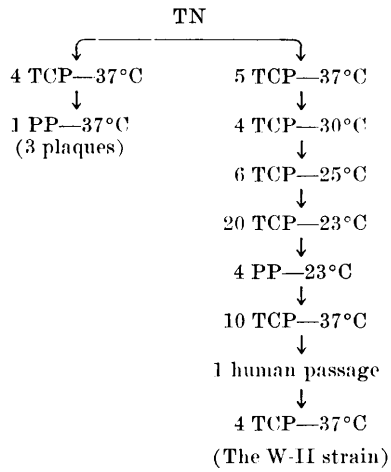

FIG. 1

TCP = Serial passages in monkey kidney tissue culture tubes.

PP = Serial single plaque passages on monkey kidney tissue culture monolayers.

titration at 37°C and 40°C. The MEF-1 strain—a virulent rct/40+ strain—was included as a control. The fifth passage of the TN strain in monkey cells maintained at 37°C yielded a pool with limited but measurable replication capacity at 40°C (Table II). Ten passages at reduced incubation temperatures, 4 at 30°C and 6 at 25°C, failed to affect appreciably the strain's growth at 40°C, but were accompanied by a marked increase in its growth potential at 25°C. Continued passages in this series led eventually to the W-II strain which had little replication potential at 40°C. In contrast to the W-II strain, the MEF-1 virus pool replicated at 40°C almost to the same extent as at 37°C.

TABLE II. Reproductive Capacity of the W-II Strain, Several W-II Progenitor Strains and the MEF-1 Strain at 25°C, 37°C, and 40°C.

Virus strain	Passages in monkey kidney tissue culture	Log_{10} TCD_{50} 25°C	37°C	40°C
TN	5 at 37°	<2.5	7.5	3.5
TN	5 at 37° 4 at 30° 6 at 25°	6.5	6.7	2.7
W-II			5.9*	1.7*
MEF-1			7.5	7.0

* Titers are an avg obtained in 5 separate experiments.

Infectivity of the W-II strain for man and its stability during human intestinal passage. Twenty-two infants whose ages ranged from 3-30 days were fed the W-II strain. Eight of the infants were fed a W-II vaccine produced in MK cells. The remaining 14 infants were fed virus that had undergone further passages in human diploid cells (WI-26) (11). Poliovirus vaccines produced in human diploid cells have been used in several previous vaccination studies(12). To produce the WI-26 pool of the W-II strain, the monkey kidney adapted vaccine pool was plaque-passed 3 times in the human diploid cell line by technics outlined elsewhere(12). A plaque picked at random from the third passage was serially passed 4 times in bottle cultures of human diploid cells to produce a large vaccine pool. The dose of virus fed varied from $10^{3.5}$ to $10^{5.7}$ TCD_{50}. Stools were collected twice weekly and processed as described previously(13). Virus isolated from the feces 3 to 25 days after ingestion of the vaccine were passed in MKTC tubes at 37°C. The resulting first passage pools were titrated at 37°C and 40°C. The results, summarized in Table III, indicated that none of the 22 isolates had altered in rct/40 character.

Discussion. This paper reports the development of type 2 attenuated poliovirus, designated W-II, derived from the mouse adapted TN vaccine strain after serial passages in MKTC systems that were maintained at low incubation temperatures. The passage series included 5 passages in culture tubes maintained at 37°C, 4 passages at 30°, 6 passages at 25° and 20 passages at 23°C. This series was followed by 4 plaque passages at 23°C, 10 rapid passages at 37°C, 1 human passage and 4 additional passages at 37°C in MKTC tubes. The pool obtained after these passages, the W-II strain, had no capacity to grow at 40°C and was not neurovirulent for monkeys injected intracerebrally. These results were in contrast to those obtained with the initial MKTC passages of the TN strain, which had a slight growth capacity at 40°C and caused extensive pathology in monkeys. Thus, the cold-passage series had led to a marked reduction in pathogenicity of the tissue culture adapted virus pool.

TABLE III. Reproductive Capacity at 37°C and 40°C of Fecal Virus Isolated from Infants Fed the W-II Strain.

Subject No.	Source of vaccine: Cell line	Dose fed $\log_{10}$ TCD_{50}	Virus isolated post-vaccination day	$\log_{10}$ TCD_{50}		
				37°C	40°C	37°C – 40°C
1	HDC	5.5	8	6.7	2.5	4.2
2	"	5.5	3	7.2	0	7.2
3	"	4.5	6	5.2	0	5.2
4	"	4.5	7	5.7	1.7	4.0
5	"	4.5	7	6.2	3.2	3.0
6	"	4.5	4	5.7	0	5.7
7	"	4.5	5	6.7	1.7	5.0
8	"	4.5	13	>6.5	3.5	>3.0
9	"	4.5	6	6.7	3.2	3.5
10	"	4.5	6	5.7	0	5.7
11	"	4.5	7	7.2	0	7.2
12	"	5.5	5	>6.5	4.2	>2.3
13	"	4.5	8	6.5	0	6.5
14	"	3.5	7	7.2	0	7.2
15	MK	6.7	25	7.7	2.7	5.0
16	"	5.2	5	7.5	0	7.5
17	"	5.2	10	6.7	3.5	3.2
18	"	5.2	8	7.5	0	7.5
19	"	5.2	4	6.5	0	6.5
20	"	5.2	6	6.5	2.2	4.3
21	"	5.7	7	6.7	0	6.7
22	"	5.7	11	7.5	3.5	4.0

The genetic stability of the W-II strain was demonstrated by study of the rct/40 character after passage of the virus through the intestinal tract of a number of infants fed different quantities of the strain. The excreted virus had little or no capacity to replicate in tissue cultures maintained at 40°C (rct/40– character). These results were in contrast to the instability of the original TN strain(6). Additional investigations are underway to determine in field studies the infectivity and antigenicity of the W-II strain.

Thus, in 2 instances, the establishment of the W-II and the WM-III strains, passage and clone selection at low temperatures have resulted in modification of existing strains to yield virus with improved vaccine characteristics. In each passage series there was a marked reduction in monkey neuropathogenicity and an improvement in strain stability(1). Dubes and Wenner(2) have observed a marked reduction in neuropathogenicity following passage of several strains at low temperatures of incubation, which appeared to be related to deadaptation to growth at 37°C.

In another study(14) strains that were rct/40+ were serially passed 14-16 times in culture tubes maintained at 23°C. These strains lost most of their capacity to replicate at 40°C. Thus, in several instances, passage at low temperatures of incubation has had a marked effect upon strain characteristics and the direction of these effects led to strains with improved potential as vaccines.

Summary. The modification of a type 2 attenuated poliovirus strain is described. Starting with the mouse adapted TN strain, passage and clone selection at low temperatures of incubation were employed to yield virus (the W-II strain) with reduced pathogenicity for monkeys and good stability during human intestinal passage.

We wish to thank the following individuals for help in obtaining infants for vaccination, and supervising their care: Donald Cornely, M.D., Paul Gyorgy, M.D., Jane Sitnek, R.N., Walter Omans, M.D., and Elizabeth Davies, R.N., Philadelphia General Hospital; and Agnes Flack, M.D., and Ruth Lorenzo, R.N., Clinton Farms. Barbara Cohen and Jean LaLiberte assisted with laboratory work. Dr. George Jervis performed histological examinations of the monkey central nervous system. Dr. Joseph Pagano made several important suggestions regarding the manuscript.

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Received October 22, 1962. P.S.E.B.M., 1963, v112.

Oncogenic Effect of Methylcholanthrene in New-Born Germfree Mice.* (28009)

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Adenomas have appeared in the lungs of mice following inoculations with urethane and other carcinogenic chemical compounds(1-4). The mechanisms whereby such pulmonary neoplasms developed from remotely inoculated carcinogenic stimuli are not clear. Kelly and O'Gara(5) induced pulmonary adenomas rapidly by inoculating methylcholanthrene (MCA) subcutaneously into new-born mice. The response to MCA was quantitated by counting the tumors on and in each sectioned lung as observed by low-power microscopy.

Investigators have demonstrated clearly the involvement of viruses in tumor formation: some, such as leukemia, polyoma, papilloma, avian lymphoma, myeloblastosis, and sarcoma viruses exert a direct oncogenic effect on the host cell(6). Other viruses (adenovirus 12 and simian virus 40) are associated primarily with non-neoplastic lesions, but under special conditions they do elicit tumorous lesions in other species of animals(7,8). Virus-like inclusions have been observed in transplanted tumor cells of animals, which were initiated by carcinogenic chemical compounds (9,10); however, the role of such inclusions in tumor formation is uncertain. Perhaps the chemically-induced undifferentiated tumor cells provide a milieu more suitable for virus propagation than cells elsewhere in the body, and thus they manifest a selective proclivity for such cells.

Viruses can induce tumors in animals; however, there is uncertainty whether, as a group, they are the exclusive cause of such lesions. Tumors have been induced in rodents by numerous well-defined chemical agents, by physical agents, and by induced endocrine imbalances(11). All of the experiments described to date were performed in conventional animals. Since such animals are now known to harbor a wide range of viruses, some being carcinogenic, the mechanism(s) of oncogenesis by the above noted non-viral agents need clarification. The most direct approach to this problem would require the use of virus-free animals. Mice have been propagated and maintained in Lobund Laboratory under germfree conditions through 19 serial genera-

* Supported by funds from U. S. Public Health Service, and Cancer Societies of St. Joseph, Elkhart, and LaPorte Counties.

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